Application for U.S. Letters Patent Entitled

MAMMALIAN CHEMOKINES; RECEPTORS; REAGENTS; USES

continuation-in-part of 09/122,585 filed July 24, 1998, which claims priority from provisional application 60/053,693

by Inventors:

Joseph A. HEDRICK
Bernhard HOMEY
Alain VICARI
Monica ZEPEDA
Albert ZLOTNIK

DNAX Research Institute
901 California Avenue
Palo Alto, CA 94304-1104
Telephone: (650) 852-9196
Facsimile: (650) 496-1200

Attorney Docket No. DX0757K
2790-0757.20

UTILITY PATENT APPLICATION

5

MAMMALIAN CHEMOKINES; RECEPTORS; REAGENTS; USES

10   INVENTORS:

Joseph A. Hedrick, a citizen of the United States,
        residing at 52-08 Quail Ridge Drive; Plainsboro, New
        Jersey 08536;

15

Bernhard Homey, a citizen of Germany, residing at 2161
        Princeton Street, Apt. 3; Palo Alto, California
        94306;

20   Alain Vicari, a citizen of France, residing at 6, rue
        Juiverie; 69005 Lyon, FRANCE;

Monica Zepeda, a citizen of the United States, residing
        at 4247 Calle Mejillones; San Diego, California
25   92130; and

Albert Zlotnik; a citizen of the United States; residing
        at 507 Alger Drive; Palo Alto, California 94306.

30

35   DNAX Research Institute
        901 California Avenue
        Palo Alto, California 94304-1104
        Tel: (650)852-9196
        Fax: (650)496-1200

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 09/122,585, filed July 24, 1998, from which priority is claimed pursuant to 35 USC §120, which

10 application is related to U.S. Provisional Application Serial No. 60/053,693, filed July 25, 1997, from which priority is claimed pursuant to 35 USC §119(e)(1), which applications are incorporated herein by reference in their entireties.

15 ## FIELD OF THE INVENTION

The present invention relates to compositions related to proteins which function in controlling physiology, development, and/or differentiation of mammalian cells. In particular, it provides proteins which are implicated in the

20 regulation of physiology, development, differentiation, or function of various cell types, e.g., chemokines, 7 transmembrane receptors, reagents related to each, e.g., antibodies or nucleic acids encoding them, and uses thereof.

25 ## BACKGROUND OF THE INVENTION

The circulating component of the mammalian circulatory system comprises various cell types, including red and white blood cells of the erythroid and myeloid cell lineages. See, e.g., Rapaport (1987) Introduction to Hematology (2d ed.)

30 Lippincott, Philadelphia, PA; Jandl (1987) Blood: Textbook of Hematology, Little, Brown and Co., Boston, MA.; and Paul (ed.) (1993) Fundamental Immunology (3d ed.) Raven Press, N.Y.

For some time, it has been known that the mammalian immune response is based on a series of complex cellular

35 interactions, called the "immune network." Recent research has provided new insights into the inner workings of this network. While it remains clear that much of the response does, in fact, revolve around the network-like interactions of

lymphocytes, macrophages, granulocytes, and other cells, immunologists now generally hold the opinion that soluble proteins, known as lymphokines, cytokines, or monokines, play a critical role in controlling these cellular interactions.

5   Thus, there is considerable interest in the isolation, characterization, and mechanisms of action of cell modulatory factors, an understanding of which should lead to significant advancements in the diagnosis and therapy of numerous medical abnormalities, e.g., immune system and other disorders.

10   Lymphokines apparently mediate cellular activities in a variety of ways. They have been shown to support the proliferation, growth, and differentiation of the pluripotential hematopoietic stem cells into vast numbers of progenitors comprising diverse cellular lineages making up a

15 complex immune system. These interactions between the cellular components are necessary for a healthy immune response. These different cellular lineages often respond in a different manner when lymphokines are administered in conjunction with other agents.

20   The chemokines are a large and diverse superfamily of proteins. The superfamily is subdivided into two classical branches, based upon whether the first two cysteines in the chemokine motif are adjacent (termed the "C-C" branch), or spaced by an intervening residue ("C-X-C"). A more recently

25 identified branch of chemokines lacks two cysteines in the corresponding motif, and is represented by the chemokines known as lymphotactins. Another recently identified branch has three intervening residues between the two cysteines, e.g., CX3C chemokines. See, e.g., Schall and Bacon (1994)

30 Current Opinion in Immunology 6:865-873; and Bacon and Schall (1996) Int. Arch. Allergy & Immunol. 109:97-109.

    The chemokine receptors are typically members of the superfamily of G-protein coupled (or linked) receptors (GPCR, or GPLR). As a class, these receptors are integral membrane

35 proteins characterized by amino acid sequences which contain seven hydrophobic domains. See, e.g., Ruffolo and Hollinger

(eds. 1995) <u>G-Protein Coupled Transmembrane Signaling Mechanisms</u> CRC Press, Boca Raton, FL; Watson and Arkinstall (1994) <u>The G-Protein Linked Receptor FactsBook</u> Academic Press, San Diego, CA; Peroutka (ed. 1994) <u>G Protein-Coupled Receptors</u>

5   CRC Press, Boca Raton, FL; Houslay and Milligan (1990) <u>G-Proteins as Mediators of Cellular Signaling Processes</u> Wiley and Sons, New York, NY; and Dohlman, et al. (1991) <u>Ann. Rev. Biochem.</u> 60:653-688. These hydrophobic domains are predicted to represent transmembrane spanning regions of the proteins.

10   These GPCRs are found in a wide range of organisms and are typically involved in the transmission of signals to the interior of the cell, e.g., through interaction, e.g., with heterotrimeric G-proteins. They respond to a wide and diverse range of agents including lipid analogs, amino acid

15   derivatives, small peptides, and other molecules.

        The presumed transmembrane segments are typically 20-25 amino acids in length. Based upon models and data on bacteriorhodopsin, these regions are predicted to be α-helices and be oriented to form a ligand binding pocket. See, e.g.,

20   Findley, et al. (1990) <u>Trends Pharmacol. Sci.</u> 11:492-499. Other data suggest that the amino termini of the proteins are extracellular, and the carboxy termini are intracellular. See, e.g., Lodish, et al. (1995) <u>Molecular Cell Biology</u> 3d ed., Scientific American, New York; and Watson and Arkinstall

25   (1994) <u>The G-Protein Linked Receptor FactsBook</u> Academic Press, San Diego, CA. Phosphorylation cascades have been implicated in the signal transduction pathway of these receptors.

        Although the full spectrum of biological activities mediated by these 7 transmembrane receptors has not been fully

30   determined, chemoattractant effects are recognized. Chemokine receptors are notable members of the GPCR family. See, e.g., Samson, et al. (1996) <u>Biochemistry</u> 35:3362-3367; and Rapport, et al. (1996) <u>J. Leukocyte Biology</u> 59:18-23. The best known biological functions of these chemokine molecules relate to

35   chemoattraction of leukocytes. However, new chemokines and receptors are being discovered, and their biological effects

on the various cells responsible for immunological responses
are topics of continued study.

        Many factors have been identified which influence the
differentiation process of precursor cells, or regulate the
5   physiology or migration properties of specific cell types.
These observations indicate that other factors exist whose
functions in immune function were heretofore unrecognized.
These factors provide for biological activities whose spectra
of effects may be distinct from known differentiation or
10   activation factors. The absence of knowledge about the
structural, biological, and physiological properties of the
regulatory factors which regulate cell physiology *in vivo*
prevents the modulation of the effects of such factors.

        In addition, other factors exist whose functions in
15   hematopoiesis, neural function, immune development, and
leukocyte trafficking were heretofore unrecognized. These
receptors mediate biological activities whose spectra of
effects are distinct from known differentiation, activation,
or other signaling factors. The absence of knowledge about
20   the structural, biological, and physiological properties of
the receptors which regulate cell physiology, development, or
function prevents the modification of the effects of such
factors.

        Thus, medical conditions where regulation of the
25   development or physiology of relevant cells is required remain
unmanageable.

SUMMARY OF THE INVENTION

30   The present invention is based, in part, upon the
discovery of new genes encoding various chemokines, e.g.,
those designated CXC N4; or 7 transmembrane receptors, e.g.,
those designated DNAXCCR10, which encode rodent receptors; and
BLRx, which encode primate receptors. Each GPCR gene encodes
35   a polypeptide exhibiting structural and/or sequence homology
to 7 transmembrane receptors. Such receptors are typically G-
protein coupled (or linked) receptors (GPCR or GPLR), though

the complete set of ligands for each has not yet been identified.

The invention also provides mutations (muteins) of the respective natural sequences, fusion proteins, chemical

5   mimetics, antibodies, and other structural or functional analogs. It is also directed to isolated nucleic acids, e.g., genes encoding respective proteins of the invention. Various uses of these different protein, antibody, or nucleic acid compositions are also provided.

10   The present invention provides a composition selected from the group of: a substantially pure antigenic polypeptide comprising sequence from a CXC N4, a DNAXCCR10, or BLRx; a binding composition comprising an antigen binding portion of an antibody specific for binding to such an antigenic

15   polypeptide; a nucleic acid encoding such an antigenic polypeptide; and a fusion protein comprising at least two non-overlapping segments of at least 10 amino acids of such an antigenic polypeptide.

In certain embodiments of the antigenic polypeptide, it

20   is from a warm blooded animal, e.g., a rodent or primate; it comprises a sequence of Figures 1-5; it exhibits a post-translational modification pattern distinct from a natural form of said polypeptide; it is detectably labeled; or it is made by expression of a recombinant nucleic acid. In other

25   embodiments, a sterile form is provided, including, e.g., composition comprising the polypeptide and an acceptable carrier. A detection kit comprising a compartment or container holding such an antigenic polypeptide is also provided.

30   In other binding composition forms, e.g., antibody embodiments, the polypeptide is a mouse or human protein; the antibody is raised against a peptide sequence of Figures 1-5; the antibody is a monoclonal antibody; the binding composition is fused to a heterologous protein, or is detectably labeled.

35   An alternative embodiment is a binding compound comprising an antigen binding fragment of the antibody described. Also provided is a detection kit comprising such a binding compound. With the antibodies are provided methods of

purifying a polypeptide using the binding compound or antibody to specifically separate the polypeptides from others, or for detection, e.g., immunohistochemistry or immunoprecipitation.

Nucleic acid embodiments are provided, e.g., where the
5 nucleic acid is in an expression vector and: encodes a polypeptide from a mouse or human; comprises a sequence of a mature protein of Figures 1-5; or comprises a deoxyribonucleic acid nucleotide. The invention also provides a kit with such nucleic acids, e.g., which include PCR primers for amplifying
10 such sequences.

With nucleic acids are provided fusion proteins, comprising: a sequence of Figures 1-5; and/or sequence of another chemokine or 7 transmembrane receptor, as appropriate. Also provided is a cell comprising a recombinant nucleic acid,
15 as described, and methods of producing a polypeptide comprising expressing the nucleic acid in an expression system.

Other embodiments include methods of modulating physiology or development of a cell, or treating a disorder,
20 with a step of contacting that cell with a composition comprising an agonist or antagonist of the chemokine or receptor. Ordinarily, the cell is a neuron, macrophage, a lymphocyte, or a skin cell, such as found in the epidermis or dermis. Various physiological effects to be modulated include
25 a cellular calcium flux, a chemoattractant response, cellular morphology modification responses, phosphoinositide lipid turnover, an antiviral response, or a proliferative response.

Yet further embodiments are directed to a method comprising administering to a subject in need thereof an
30 effective amount of an agent that modulates the expression or activity of BLRx or fragments thereof. In a further embodiment, the method of treatment or prevention comprises administering to a subject in need thereof an effective amount of an agent that enhances or decreases the activity or
35 expression of BLRx or fragments thereof. The agent can be, for example, a polynucleotide encoding BLRx, a polynucleotide that includes a transcriptional or translational regulator, an antibody that specifically binds to BLRx, an antisense

oligonucleotide having a sequence that binds to a sequence encoding BLRx, or a small molecule inhibitor.

The present methods can be directed to the treatment of various disorders, including, for example, wound healing,
5   proliferative disorders, fibrotic disorders, sclerotic disorders, cancer, and angiogenesis.

In another aspect, the present invention encompasses pharmaceutical compositions comprising an effective amount of an agent that modulates the expression or activity of BLRx and
10   a suitable carrier. The agent can be a BLRx agonist or antagonist, for example, or can comprise BLRx and a suitable carrier.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of
15   the disclosure herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 (SEQ ID NOS:1 and 2) depicts the partial nucleotide sequence (5' to 3') and corresponding amino acid
20   sequence of a rodent embodiment of a chemokine designated CXC N4 (SEQ ID NO:1). The predicted signal cleavage site is at approximately the gly19-gln20 peptide bond. The CXC motif corresponds to residues cys27 through cys29.

Figures 2A-2B (SEQ ID NOS:3 and 4)show the nucleotide
25   sequence (5' to 3') and corresponding amino acid sequence of a human embodiment of a chemokine receptor similar to one designated GPCR W. Nucleotides 1106 and 1139 differ from the previously reported human and canine sequences.

Figures 3A-3C (SEQ ID NOS:7 and 8) show the nucleotide
30   sequence (5' to 3') and corresponding amino acid sequence of a human embodiment of a primate 7 transmembrane receptor family member, designated BLRx. Ambiguities in the sequence are as follows: residue 1462 may be G/T; 1473 may be A/C; 1490 may be A/C/G/T; and 1495 may be A/T. Only the first is in the coding
35   sequence, thus residue 193 may be gly or arg.

Figures 4A-4B (SEQ ID NOS:9 and 10) show a mouse DNAXCCR10 nucleotide sequence segment and the corresponding amino acid sequence.

Figure 5 (SEQ ID NOS:5 and 6) shows a partial mouse
5   DNAXCCR10 nucleotide sequence segment and corresponding amino acid sequence.

Figure 6 shows expression levels of BLRx in various tissues and cell types. Expression levels were normalized and expressed as femtograms mRNA per 50 ng total cDNA. Acronyms
10 are as follows: DMEC = dermal microvascular endothelial cell; DMEC N = untreated dermal microvascular endothelial cell; FB = fibroblast; FB N = untreated fibroblast; MC = melanocyte; MC N = untreated melanocyte; and KC = keratinocyte.

Figure 7 shows BLRx expression during various phases of
15 wound healing.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

All references cited herein are incorporated herein by reference to the same extent as if each individual publication
20 or patent application was specifically and individually indicated to be incorporated by reference.

I.   General

The present invention provides DNA sequences encoding
25 various mammalian proteins, including chemokines, or which exhibit structural properties characteristic of a 7 transmembrane receptor. See, e.g., Ruffolo and Hollinger (eds. 1995) G-Protein Coupled Transmembrane Signaling Mechanisms CRC Press, Boca Raton, FL; Watson and Arkinstall
30 (1994) The G-Protein Linked Receptor FactsBook Academic Press, San Diego, CA; Peroutka (ed. 1994) G Protein-Coupled Receptors CRC Press, Boca Raton, FL; Houslay and Milligan (1990) G-Proteins as Mediators of Cellular Signaling Processes Wiley and Sons, New York, NY. Certain mouse and human embodiments
35 are described herein.

Among the many types of ligands which mediate biology via these receptors are chemokines and certain proteases. Chemokines play an important role in immune and inflammatory responses by inducing migration and adhesion of leukocytes.

5   See, e.g., Schall (1991) Cytokine 3:165-183; and Thomson (ed.) The Cytokine Handbook Academic Press, NY. Chemokines are secreted by activated leukocytes and act as a chemoattractant for a variety of cells which are involved in inflammation. Besides chemoattractant properties, chemokines have been shown

10   to induce other biological responses, e.g., modulation of second messenger levels such as Ca^{++}; inositol phosphate pool changes (see, e.g., Berridge (1993) Nature 361:315-325 or Billah and Anthes (1990) Biochem. J. 269:281-291); cellular morphology modification responses; phosphoinositide lipid

15   turnover; possible antiviral responses; and others. Thus, the chemokines provided herein may, alone or in combination with other therapeutic reagents, have advantageous combination effects.

Moreover, there are reasons to suggest that chemokines

20   may have effects on other cell types, e.g., attraction or activation of monocytes, dendritic cells, T cells, eosinophils, and/or perhaps on basophils and/or neutrophils. They may also have chemoattractive effects on various neural cells including, e.g., dorsal root ganglia neurons in the

25   peripheral nervous system and/or central nervous system neurons.

G-protein coupled receptors, e.g., chemokine receptors, are important in the signal transduction mechanisms mediated by their ligands. They are useful markers for distinguishing

30   cell populations, and have been implicated as specific receptors for retroviral infections.

The chemokine superfamily was classically divided into two groups exhibiting characteristic structural motifs, the Cys-X-Cys (C-X-C) and Cys-Cys (C-C) families. These were

35   distinguished on the basis of a single amino acid insertion between the NH-proximal pair of cysteine residues and sequence

similarity. Typically, the C-X-C chemokines, i.e., IL-8 and

MGSA/Gro-α act on neutrophils but not on monocytes, whereas

the C-C chemokines, i.e., MIP-1α and RANTES, are potent

chemoattractants for monocytes and lymphocytes but not

5 neutrophils. See, e.g., Miller, et al. (1992) Crit. Rev.

Immunol. 12:17-46. A recently isolated chemokine,

lymphotactin, does not belong to either group and may

constitute a first member of a third chemokine family, the C

family. Lymphotactin does not have a characteristic CC or CXC

10 motif, and acts on lymphocytes but not neutrophils and

monocytes. See, e.g., Kelner et al. (1994) Science 266:1395-

1399. This chemokine defines a new C-C chemokine family.

Even more recently, another chemokine exhibiting a CX3C motif

has been identified, which establishes a fourth structural

15 class.

The present invention provides additional chemokine

reagents, e.g., nucleic acids, proteins and peptides,

antibodies, etc., related to the newly discovered chemokine

designated rodent CXC N4.

20 In other embodiments, the invention provides genes

encoding novel G-protein coupled receptors, designated mouse

DNAXCCR10 and primate BLRx. Their ligands have not yet

specifically been identified, but the DNAXCCR10 responds to

binding to the chemokine MIP-3α. See Hieshima, et al. (1997)

25 J. Biol. Chem. 272:5846-5853; Hromas, et al. (1997) Blood

89:3315-3322; and Baba, et al. (1997) J. Biol. Chem.

272:14893-14898. The receptors exhibit structural features

typical of known 7 transmembrane spanning receptors, which

receptors include chemokine receptors. The receptors may

30 exhibit properties of binding many different cytokines at

varying specificities (shared or promiscuous binding

specificity) or may exhibit high affinity for one (specific)

or a subset (shared) of chemokines. Alternatively, the

ligands may be other molecules, including molecules such as

35 epinephrine, serotonin, or glucagon.

The described chemokines or receptors should be important

for mediating various aspects of cellular, organ, tissue, or organismal physiology or development.

II.   Purified Chemokines; Receptors

5   Nucleotide and derived amino acid sequences of a novel rodent CXC chemokine, e.g., from mouse, designated CXC N4 are shown in Figure 1 (SEQ ID NOS:1 and 2). The gene encodes a novel protein exhibiting structure and motifs characteristic of a chemokine. Its closest reported chemokines are the mouse

10   SDF-1, IP-10, and MIG chemokines. See, e.g., Aiuti, et al. (1997) J. Exp. Med. 185:111-120; Loetscher, et al. (1996) J. Exp. Med. 184:963-969; and Sgadari, et al. (1997) Blood 89:2635-2643.

         Nucleotide and derived amino acid sequences of a novel

15   primate GPCR, e.g., from human, designated DNAXCCR10, are shown in Figures 2A-2B (SEQ ID NOS:3 and 4). Sequence analysis shows closest sequence homology to a human GPCR designated GPCR W (accession number L42324) or to a canine GPCR W (accession number L42326); and to a mouse sequence

20   (accession number AA423677). As indicated above, the receptor responds to binding with chemokine ligand MIP-3α, which immediately allows for screening for ligands since a positive control is available.

         Nucleotide and derived amino acid sequences of a rodent

25   GPCR, e.g., from mouse, DNAXCCR10, are shown in Figures 4A-4B (SEQ ID NOS:9 and 10). A partial mouse sequence is shown in Figure 5 (SEQ ID NOS:5 and 6)

         Nucleotide and derived amino acid sequences of a novel primate, e.g., from human, 7 transmembrane receptor family

30   member, designated BLRx herein, are shown in Figures 3A-3C (SEQ ID NOS:7 and 8). Sequence analysis shows sequence homology to various GPCR family members, particularly the bovine gustative receptor. See Forster, et al. (1996) Cell 13:1037-1047.

35   Certain general descriptions of physical properties of polypeptides, nucleic acids, and antibodies, where directed to

one embodiment clearly are usually applicable to other
chemokines or receptors described herein.

These amino acid sequences, provided amino to carboxy,
are important in providing sequence information on the
5   chemokine ligand or receptor, allowing for distinguishing the
protein from other proteins, particularly naturally occurring
versions. Moreover, the sequences allow preparation of
peptides to generate antibodies to recognize and distinguish
such segments, and allow preparation of oligonucleotide
10   probes, both of which are strategies for isolation, e.g.,
cloning, of genes encoding such sequences, or related
sequences, e.g., natural polymorphic or other variants,
including fusion proteins. Similarities of the chemokines
have been observed with other cytokines. See, e.g.,
15   Bosenberg, et al. (1992) Cell 71:1157-1165; Huang, et. al.
(1992) Molecular Biology of the Cell 3:349-362; and Pandiella,
et al. (1992) J. Biol. Chem. 267:24028-24033. Likewise for
the GPC receptors.

As used herein, the term "CXC N4" shall encompass, when
20   used in a protein context, a protein having an amino acid
sequence as shown in Figure 1. Similarly, by the term "BLRx"
is meant a protein with an amino acid sequence as depicted in
Figures 3A-3C herein. Likewise, the term "DNAXCCR10" refers
to a protein having the amino acid sequence depicted in
25   Figures 2A-2B. Preferably, such sequences will be the native
or natural sequence, e.g., a sequence corresponding to the
native sequence from a mammal, such as a primate, rodent, etc.
The invention also embraces a polypeptide comprising a
significant fragment of such protein. The invention also
30   encompasses a counterpart polypeptide from another mammalian
species, e.g., which exhibits similar sequence, and is more
homologous in the native coding sequence than other genes from
the species. Typically, in the case of homologs of the
reference sequence, the molecule will interact with its
35   specific binding components, such as antibodies which bind the
sequence, its receptor if the molecule is a chemokine, or the
appropriate ligand, if the molecule is a receptor. These
binding components, e.g., antibodies, typically bind to the

chemokine with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at better than about 3 nM. Similar concepts apply to the binding components which recognize

5   mammalian embodiments for the GPCRs DNAXCCR10 and BLRx.

The term "polypeptide" as used herein includes a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino

10   acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in

15   particularly preferred embodiments, at least about 30 or more amino acids, e.g., about 35, 40, 45, 50, etc. Similar proteins will likely comprise a plurality of such segments. Such fragments may have ends which begin and/or end at virtually all positions, e.g., beginning at residues 1, 2, 3,

20   etc., and ending at, e.g., 54, 53, 52, etc., in all combinatorial pairs in the coding segment. Typically, the plurality will be at least two, more usually at least three, and preferably 5, 7, or even more. While the length minima are provided, longer lengths, of various sizes, may be

25   appropriate, e.g., one of length 7, and two of length 12.

Particularly interesting peptides have ends corresponding to structural domain boundaries, e.g., intracellular or extracellular loops of the receptor embodiments. Such peptides will typically be immunogenic peptides, e.g.,

30   including peptides of at least 12, 14, 16, etc., residues or may be concatenated to generate larger polypeptides. Short peptides may be attached or coupled to a larger carrier.

The term "binding composition" refers to molecules that bind with specificity to the respective chemokine or receptor,

35   e.g., in antibody-antigen interaction, or alternatively in a ligand-receptor type fashion. These compositions may be compounds, e.g., proteins, which specifically associate with the chemokine or receptor, including natural physiologically

relevant protein-protein interactions, either covalent or non-covalent. The binding composition may be a polymer, or another chemical reagent. No implication as to whether the chemokine presents a concave or convex shape in its ligand-

5   receptor interaction is necessarily represented, other than the interaction exhibit similar specificity, e.g., specific affinity. A functional analog may be a ligand with structural modifications, or may be a wholly unrelated molecule, e.g., which has a molecular shape which interacts with the

10   appropriate ligand binding determinants. The ligands may serve as agonists or antagonists of a physiological or natural receptor, see, e.g., Goodman, et al. (eds.) (1990) Goodman & Gilman's: The Pharmacological Bases of Therapeutics (8th ed.), Pergamon Press. The term expressly includes compounds

15   comprising antigen binding portions of antibodies, polyclonal or monoclonal, e.g., which specifically bind to the respective antigen.

       Substantially pure means that the protein is free from other contaminating proteins, nucleic acids, and/or other

20   biologicals typically derived from the original source organism. Purity may be assayed by standard methods, and will ordinarily be at least about 40% pure, more ordinarily at least about 50% pure, generally at least about 60% pure, more generally at least about 70% pure, often at least about 75%

25   pure, more often at least about 80% pure, typically at least about 85% pure, more typically at least about 90% pure, preferably at least about 95% pure, more preferably at least about 98% pure, and in most preferred embodiments, at least 99% pure. Analyses will typically be by weight, but may be by

30   molar amounts.

       Solubility of a polypeptide or fragment depends upon the environment and the polypeptide. Many parameters affect polypeptide solubility, including temperature, electrolyte environment, size and molecular characteristics of the

35   polypeptide, and nature of the solvent. Typically, the temperature at which the polypeptide is used ranges from about 4 °C to about 65 °C. Usually the temperature at use is greater than about 18 °C and more usually greater than about
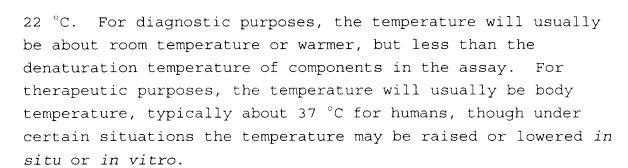
22 °C. For diagnostic purposes, the temperature will usually be about room temperature or warmer, but less than the denaturation temperature of components in the assay. For therapeutic purposes, the temperature will usually be body temperature, typically about 37 °C for humans, though under certain situations the temperature may be raised or lowered *in situ* or *in vitro*.

The electrolytes will usually approximate *in situ* physiological conditions, but may be modified to higher or lower ionic strength where advantageous. The actual ions may be modified, e.g., to conform to standard buffers used in physiological or analytical contexts.

The size and structure of the polypeptide should generally be in a substantially stable state, and usually not in a denatured state, though in certain circumstances denatured protein will be important. The polypeptide may be associated with other polypeptides in a quaternary structure, e.g., to confer solubility, or associated with lipids or detergents in a manner which approximates natural lipid bilayer interactions.

The solvent will usually be a biologically compatible buffer, of a type used for preservation of biological activities, and will usually approximate a physiological solvent. Usually the solvent will have a neutral pH, typically at least about 5, preferably at least 6, and typically less than 10, preferably less than 9, and more preferably about 7.5. On some occasions, a detergent will be added, typically a mild non-denaturing one, e.g., CHS (cholesteryl hemisuccinate) or CHAPS (3-([3-cholamido-propyl]dimethylammonio)-1-propane sulfonate), or a low enough concentration as to avoid significant disruption of structural or physiological properties of the protein.

Solubility is reflected by sedimentation measured in Svedberg units, which are a measure of the sedimentation velocity of a molecule under particular conditions. The determination of the sedimentation velocity was classically performed in an analytical ultracentrifuge, but is typically now performed in a standard ultracentrifuge. See, Freifelder

(1982) <u>Physical Biochemistry</u> (2d ed.), W.H. Freeman; and
Cantor and Schimmel (1980) <u>Biophysical Chemistry</u>, parts 1-3,
W.H. Freeman & Co., San Francisco. As a crude determination,
a sample containing a putatively soluble polypeptide is spun
5   in a standard full sized ultracentrifuge at about 50K rpm for
about 10 minutes, and soluble molecules will remain in the
supernatant. A soluble particle or polypeptide will typically
be less than about 30S, more typically less than about 15S,
usually less than about 10S, more usually less than about 6S,
10   and, in particular embodiments, preferably less than about 4S,
and more preferably less than about 3S.

     III. Physical Variants
          This invention also encompasses proteins or peptides
15   having substantial amino acid sequence homology with the amino
acid sequence of each respective chemokine or chemokine
receptor. The variants include species or polymorphic
variants.
          Amino acid sequence homology, or sequence identity, is
20   determined by optimizing residue matches, if necessary, by
introducing gaps as required. This changes when considering
conservative substitutions as matches. Conservative
substitutions typically include substitutions within the
following groups: glycine, alanine; valine, isoleucine,
25   leucine; aspartic acid, glutamic acid; asparagine, glutamine;
serine, threonine; lysine, arginine; and phenylalanine,
tyrosine. Homologous amino acid sequences are typically
intended to include natural allelic and interspecies
variations in each respective protein sequence. Typical
30   homologous proteins or peptides will have from 25-100%
homology (if gaps can be introduced), to 50-100% homology (if
conservative substitutions are included) with the amino acid
sequence of the appropriate chemokine or receptor, or any
percentage between these stated ranges. Homology measures
35   will be at least about 35%, generally at least 40%, more
generally at least 45%, often at least 50%, more often at
least 55%, typically at least 60%, more typically at least
65%, usually at least 70%, more usually at least 75%,

preferably at least 80%, and in particularly preferred embodiments, at least 85%, 90%, 95% or more. See also Needleham, et al. (1970) J. Mol. Biol. 48:443-453; Sankoff, et al. (1983) Chapter One in Time Warps, String Edits, and

5   Macromolecules: The Theory and Practice of Sequence Comparison Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI.

    Each of the isolated chemokine or GPC receptor DNAs can

10 be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications may result in novel DNA sequences which encode these proteins, their derivatives, or proteins having similar physiological, immunogenic, or

15 antigenic activity. These modified sequences can be used to produce mutant proteins or to enhance expression, or to introduce convenient enzyme recognition sites into the nucleotide sequence without significantly affecting the encoded protein sequence. Enhanced expression may involve

20 gene amplification, increased transcription, increased translation, and other mechanisms. Enhanced expression may be useful in the context of wound healing, as described further below. Such mutant receptor derivatives include predetermined or site-specific mutations of the respective protein or its

25 fragments. "Mutant chemokine" or "mutant chemokine receptor" encompasses a polypeptide otherwise falling within the homology definition of the chemokine or receptor as set forth above, but having an amino acid sequence which differs from that of the chemokine or receptor as found in nature, whether

30 by way of deletion, substitution, or insertion. These include amino acid residue substitution levels from none, one, two, three, five, seven, ten, twelve, fifteen...75 etc., and any number within this range. In particular, "site specific mutant" generally includes proteins having significant

35 homology with a protein having sequences of Figures 1-5, and as sharing various biological activities, e.g., antigenic or immunogenic, ligand binding, with those sequences, and in preferred embodiments contain a plurality, or most, of

disclosed sequences, particularly those found in various related groups of animals. As stated before, it is emphasized that descriptions are generally meant to encompass the various chemokine or receptor proteins from other members of related

5   groups, not limited to the mouse or human embodiments specifically discussed.

Although site specific mutation sites are often predetermined, mutants need not be site specific. Chemokine or receptor mutagenesis can be conducted by making amino acid

10   insertions or deletions, including with PCR mutagenesis. Substitutions, deletions, insertions, or combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy-terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed mutants can then

15   be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis or polymerase chain reaction (PCR) techniques. See also Sambrook, et al. (1989) and Ausubel, et al. (1987 and

20   Supplements). Many structural features are known about the chemokines and GPCRs which allow determination of whether specific residues are embedded into the core of the secondary or tertiary structures, or whether the residues will have relatively little effect on protein folding. Preferred

25   positions for mutagenesis are those which do not prevent functional folding of the resulting protein.

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce

30   secondary mRNA structure such as loops or hairpins. But certain situations exist where such problems are compensated. See, e.g., Gesteland and Atkins (1996) Ann. Rev. Biochem. 65:741-768.

The present invention also provides recombinant proteins,

35   e.g., heterologous fusion proteins using segments from these proteins, or antibodies. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product

of an immunoglobulin with a receptor polypeptide is a
continuous protein molecule having sequences fused in a
typical peptide linkage, typically made as a single
translation product and exhibiting properties derived from
5   each source peptide. A similar chimeric concept applies to
heterologous nucleic acid sequences.

In addition, new constructs may be made from combining
similar functional or structural domains from other proteins.
For example, ligand-binding or other segments may be "swapped"
10   between different new fusion polypeptides or fragments. See,
e.g., Cunningham, et al. (1989) Science 243:1330-1336; and
O'Dowd, et al. (1988) J. Biol. Chem. 263:15985-15992. Thus,
new chimeric polypeptides exhibiting new combinations of
specificities will result from the functional linkage of
15   ligand-binding specificities and other functional domains.
Such may be chimeric molecules with mixing or matching of the
various structural segments, e.g., the β-sheet or α-helix
structural domains for the chemokine, or receptor segments
corresponding to each of the transmembrane segments (TM1-TM7),
20   or the intracellular (cytosolic, C1-C4) or extracellular (E1-
E4) loops from the various receptor types. The C3 loop is
particularly important.

The phosphoramidite method described by Beaucage and
Carruthers (1981) Tetra. Letts. 22:1859-1862, will produce
25   suitable synthetic DNA fragments. A double stranded fragment
will often be obtained either by synthesizing the
complementary strand and annealing the strand together under
appropriate conditions or by adding the complementary strand
using DNA polymerase with an appropriate primer sequence,
30   e.g., PCR techniques.

IV. Functional Variants

The blocking of physiological response to various
embodiments of these chemokines or GPCRs may result from the
35   inhibition of binding of the ligand to its receptor, likely
through competitive inhibition. Thus, in vitro assays of the
present invention will often use isolated protein, membranes

from cells expressing a recombinant membrane associated receptor, e.g., ligand binding segments, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either binding

5   segment mutations and modifications, or ligand mutations and modifications, e.g., ligand analogs.

This invention also contemplates the use of competitive drug screening assays, e.g., where neutralizing binding compositions, e.g., antibodies, to antigen or receptor

10   fragments compete with a test compound for binding to the protein. In this manner, the antibodies can be used to detect the presence of polypeptides which share one or more antigenic binding sites of the ligand and can also be used to occupy binding sites on the protein that might otherwise interact

15   with a receptor.

Additionally, neutralizing antibodies against a specific chemokine embodiment and soluble fragments of the chemokine which contain a high affinity receptor binding site, can be used to inhibit chemokine activity in tissues, e.g., tissues

20   experiencing abnormal physiology.

"Derivatives" of chemokine proteins include amino acid sequence mutants, glycosylation variants, and covalent or aggregate conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to

25   groups which are found in chemokine amino acid side chains or at the N- or C- termini, by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives

30   of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species. Covalent

35   attachment to carrier proteins may be important when immunogenic moieties are haptens.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a

polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally

5   provide such processing, e.g., mammalian glycosylation enzymes. Deglycosylation enzymes are also contemplated. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or

10   phosphothreonine, or nucleoside or nucleotide derivatives, e.g., guanyl derivatized.

A major group of derivatives are covalent conjugates of the respective chemokine or receptor or fragments thereof with other proteins or polypeptides. These derivatives can be

15   synthesized in recombinant culture such as - or C-terminal fusions or by the use of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred chemokine derivatization sites with cross-linking agents are at free amino groups, carbohydrate

20   moieties, and cysteine residues.

Fusion polypeptides between these chemokines or receptors and other homologous or heterologous proteins, e.g., other chemokines or receptors, are also provided. Many growth factors and cytokines are homodimeric entities, and a repeat

25   construct may have various advantages, including lessened susceptibility to proteolytic cleavage. Moreover, many cytokine receptors require dimerization to transduce a signal, and various dimeric ligands or domain repeats can be desirable. Homologous polypeptides may be fusions between

30   different surface markers, resulting in, e.g., a hybrid protein exhibiting receptor binding specificity. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions of a reporter

35   polypeptide, e.g., luciferase, with a segment or domain of a ligand, e.g., a receptor-binding segment, so that the presence or location of the fused ligand, or a binding composition, may be easily determined. See, e.g., Dull, et al., U.S. Patent

No. 4,859,609. Other gene fusion partners include bacterial β-galactosidase, trpE, Protein A, β-lactamase, alpha amylase, alcohol dehydrogenase, a FLAG fusion, and yeast alpha mating factor. See, e.g., Godowski, et al. (1988) _Science_ 241:812-
5   816.

The phosphoramidite method described by Beaucage and Carruthers (1981) _Tetra. Letts._ 22:1859-1862, will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the
10   complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, guanylation,
15   sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate or guanyl groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity tags as FLAG.
20   Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook, et al. (1989) _Molecular Cloning: A Laboratory Manual_ (2d
25   ed.), Vols. 1-3, Cold Spring Harbor Laboratory. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) _J. Amer. Chem. Soc._ 85:2149-2156; Merrifield (1986) _Science_ 232: 341-347; and Atherton, et al. (1989) _Solid Phase Peptide Synthesis: A Practical Approach_, IRL Press,
30   Oxford; and chemical ligation, e.g., Dawson, et al. (1994) _Science_ 266:776-779, a method of linking long synthetic peptides by a peptide bond.

This invention also contemplates the use of derivatives of these chemokines or receptors other than variations in
35   amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally include: (1) salts, (2) side chain and terminal residue covalent modifications, and

(3) adsorption complexes, for example with cell membranes.
Such covalent or aggregative derivatives are useful as
immunogens, as reagents in immunoassays, or in purification
methods such as for affinity purification of ligands or other

5   binding ligands. For example, a chemokine antigen can be
immobilized by covalent bonding to a solid support such as
cyanogen bromide-activated Sepharose, by methods which are
well known in the art, or adsorbed onto polyolefin surfaces,
with or without glutaraldehyde cross-linking, for use in the

10   assay or purification of anti-chemokine antibodies or its
receptor. These chemokines can also be labeled with a
detectable group, for example radioiodinated by the chloramine
T procedure, covalently bound to rare earth chelates, or
conjugated to a fluorescent moiety for use in diagnostic

15   assays. Purification of chemokine, receptor, or binding
compositions may be effected by immobilized antibodies or
receptor.

     Other modifications may be introduced with the goal of
modifying the therapeutic pharmacokinetics or pharmacodynamics

20   of a target chemokine. For example, certain means to minimize
the size of the entity may improve its pharmacoaccessibility;
other means to maximize size may affect pharmacodynamics.
Similarly, changes in ligand binding kinetics or equilibrium
of a receptor may be engineered.

25   A solubilized chemokine or receptor or appropriate
fragment of this invention can be used as an immunogen for the
production of antisera or antibodies specific for the ligand,
receptor, or fragments thereof. The purified proteins can be
used to screen monoclonal antibodies or chemokine-binding

30   fragments prepared by immunization with various forms of
impure preparations containing the protein. In particular,
antibody equivalents include antigen binding fragments of
natural antibodies, e.g., Fv, Fab, or $F(ab)_2$. Purified
chemokines can also be used as a reagent to detect antibodies

35   generated in response to the presence of elevated levels of
the protein or cell fragments containing the protein, both of
which may be diagnostic of an abnormal or specific
physiological or disease condition. Additionally, chemokine

protein fragments, or their concatenates, may also serve as immunogens to produce binding compositions, e.g., antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies raised against

5   certain amino acid sequences, e.g., in Figures 1-5, or proteins containing them. In particular, this invention contemplates antibodies having binding affinity to or being raised against specific fragments, e.g., those which are predicted to lie on the outside surfaces of protein tertiary

10   structure. Similar concepts apply to antibodies specific for receptors of the invention.

        The present invention contemplates the isolation of additional closely related species variants. Southern and Northern blot analysis should establish that similar genetic

15   entities exist in other related mammals, and establish the stringency of hybridization conditions to isolate such. It is likely that these chemokines and receptors are widespread in species variants, e.g., among the rodents and the primates.

        The invention also provides means to isolate a group of

20   related chemokines or receptors displaying both distinctness and similarities in structure, expression, and function. Elucidation of many of the physiological effects of the proteins will be greatly accelerated by the isolation and characterization of distinct species variants of the ligands.

25   Related genes found, e.g., in various computer databases will also be useful, in many instances, for similar purposes with structurally related proteins. In particular, the present invention provides useful probes or search features for identifying additional homologous genetic entities in

30   different species.

        The isolated genes will allow transformation of cells lacking expression of a corresponding chemokine or receptor, e.g., either species types or cells which lack corresponding antigens and exhibit negative background activity. Expression

35   of transformed genes will allow isolation of antigenically pure cell lines, with defined or single specie variants. This approach will allow for more sensitive detection and discrimination of the physiological effects of chemokine or

receptor proteins.   Subcellular fragments, e.g., cytoplasts or
membrane fragments, can be isolated and used.

Dissection of critical structural elements which effect
the various differentiation functions provided by ligands is
5   possible using standard techniques of modern molecular
biology, particularly in comparing members of the related
class. See, e.g., the homolog-scanning mutagenesis technique
described in Cunningham, et al. (1989) Science 243:1339-1336;
and approaches used in O'Dowd, et al. (1988) J. Biol. Chem.
10   263:15985-15992; and Lechleiter, et al. (1990) EMBO J. 9:4381-
4390.

In addition, various segments can be substituted between
species variants to determine what structural features are
important in both receptor binding affinity and specificity,
15   as well as signal transduction. An array of different
chemokine or receptor variants will be used to screen for
variants exhibiting combined properties of interaction with
different species variants.

Intracellular functions would probably involve segments
20   of the receptor which are normally accessible to the cytosol.
However, ligand internalization may occur under certain
circumstances, and interaction between intracellular
components and "extracellular" segments may occur. The
specific segments of interaction of a particular chemokine
25   with other intracellular components may be identified by
mutagenesis or direct biochemical means, e.g., cross-linking
or affinity methods. Structural analysis by crystallographic
or other physical methods will also be applicable. Further
investigation of the mechanism of signal transduction will
30   include study of associated components which may be isolatable
by affinity methods or by genetic means, e.g., complementation
analysis of mutants.

Further study of the expression and control of the
various chemokines or receptors will be pursued. The
35   controlling elements associated with the proteins may exhibit
differential developmental, tissue specific, or other
expression patterns. Upstream or downstream genetic regions,
e.g., control elements, are of interest. Differential

splicing of message may lead to membrane bound forms, soluble
forms, and modified versions of ligand.

Structural studies of the proteins will lead to design of
new ligands or receptors, particularly analogs exhibiting
5   agonist or antagonist properties on the receptor. This can be
combined with previously described screening methods to
isolate ligands exhibiting desired spectra of activities.

Expression in other cell types will often result in
glycosylation differences in a particular chemokine or
10   receptor. Various species variants may exhibit distinct
functions based upon structural differences other than amino
acid sequence. Differential modifications may be responsible
for differential function, and elucidation of the effects are
now made possible.

15   Thus, the present invention provides important reagents
related to a physiological ligand-receptor interaction.
Although the foregoing description has focused primarily upon
the mouse and human embodiments of the chemokines or receptors
specifically described, those of skill in the art will
20   immediately recognize that the invention provides other
counterparts, e.g., from related species, rodents or primates.

V.   Antibodies

Antibodies can be raised to these chemokines or
25   receptors, including species or polymorphic variants, and
fragments thereof, both in their naturally occurring forms and
in their recombinant forms. Additionally, antibodies can be
raised to chemokines or receptors in either their active or
inactive forms, or in their native or denatured forms. Anti-
30   idiotypic antibodies are also contemplated.

Antibodies, including binding fragments and single chain
versions, against predetermined fragments of the ligands can
be raised by immunization of animals with concatemers or
conjugates of the fragments with immunogenic proteins.
35   Monoclonal antibodies are prepared from cells secreting the
desired antibody. These antibodies can be screened for
binding to normal or defective chemokines or receptors, or
screened for agonistic or antagonistic activity. These

monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, typically at least about 10 μM, more typically at least about 30 μM, preferably at least about 10 μM, and more preferably at least
5   about 3 μM or better.

The antibodies, including antigen binding fragments, of this invention can have significant preparative, diagnostic, or therapeutic value. They can be useful to purify or label the desired antigen in a sample, or may be potent antagonists
10 that bind to ligand and inhibit binding to receptor or inhibit the ability of a ligand to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to, or as fusion proteins with, toxins or radionuclides so that when the antibody binds to antigen, a
15 cell expressing it, e.g., on its surface via receptor, is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker, and may effect drug targeting. Antibodies to receptors may be more easily used to block ligand binding
20 and/or signal transduction.

The antibodies of this invention can also be useful in diagnostic or reagent purification applications. As capture or non-neutralizing antibodies, they can be screened for ability to bind to the chemokines or receptors without
25 inhibiting ligand-receptor binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying chemokine or receptors, e.g., in immunoassays. They may be used as purification reagents in immunoaffinity columns or as
30 immunohistochemistry reagents.

Ligand or receptor fragments may be concatenated or joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. Short peptides will preferably be made as repeat structures to
35 increase size. A ligand and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See Microbiology, Hoeber Medical Division, Harper and Row,

1969; Landsteiner (1962) <u>Specificity of Serological Reactions</u>, Dover Publications, New York, and Williams, et al. (1967) <u>Methods in Immunology and Immunochemistry</u>, Vol. 1, Academic Press, New York, for descriptions of methods of preparing

5   polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin fraction is isolated.

In some instances, it is desirable to prepare monoclonal

10   antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, et al. (eds.) <u>Basic and Clinical Immunology</u> (4th ed.), Lange Medical Publications, Los Altos, CA, and references

15   cited therein; Harlow and Lane (1988) <u>Antibodies: A Laboratory Manual</u>, CSH Press; Goding (1986) <u>Monoclonal Antibodies: Principles and Practice</u> (2d ed.) Academic Press, New York; and particularly in Kohler and Milstein (1975) in <u>Nature</u> 256:495-497, which discusses one method of generating

20   monoclonal antibodies. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken, e.g., from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*.

25   The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in

30   response to a specific site recognized on the immunogenic substance. Large amounts of antibody may be derived from ascites fluid from an animal.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to

35   selection of libraries of antibodies in phage or similar vectors. See, Huse, et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," <u>Science</u> 246:1275-1281; and Ward, et al. (1989)

<u>Nature</u> 341:544-546. The polypeptides and antibodies of the
present invention may be used with or without modification,
including chimeric or humanized antibodies. Frequently, the
polypeptides and antibodies will be labeled by joining, either
5   covalently or non-covalently, a substance which provides for a
detectable signal. A wide variety of labels and conjugation
techniques are known and are reported extensively in both the
scientific and patent literature. Suitable labels include
radionuclides, enzymes, substrates, cofactors, inhibitors,
10   fluorescent moieties, chemiluminescent moieties, magnetic
particles, and the like. Patents, teaching the use of such
labels include U.S. Patent Nos. 3,817,837; 3,850,752;
3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.
Also, recombinant immunoglobulins may be produced, see
15   Cabilly, U.S. Patent No. 4,816,567; and Queen et al. (1989)
<u>Proc. Nat'l. Acad. Sci.</u> 86:10029-10033.

          The antibodies of this invention can also be used for
affinity chromatography in isolating the protein. Columns can
be prepared where the antibodies are linked to a solid
20   support, e.g., particles, such as agarose, Sephadex, or the
like, where a cell lysate may be passed through the column,
the column washed, followed by increasing concentrations of a
mild denaturant, whereby the purified chemokine protein will
be released.

25   The antibodies may also be used to screen expression
libraries for particular expression products. Usually the
antibodies used in such a procedure will be labeled with a
moiety allowing easy detection of presence of antigen by
antibody binding.

30   Antibodies raised against these chemokines or receptors
will also be useful to raise anti-idiotypic antibodies. These
will be useful in detecting or diagnosing various
immunological conditions related to expression of the
respective antigens.

35

     VI.   Nucleic Acids

          The described peptide sequences and the related reagents
are useful in isolating a DNA clone encoding these chemokines

or receptors, e.g., from a natural source. Typically, it will be useful in isolating a gene from another individual, and similar procedures will be applied to isolate genes from related species, e.g., rodents or primates. Cross

5   hybridization will allow isolation of ligand from other closely related species. A number of different approaches should be available to successfully isolate a suitable nucleic acid clone. Similar concepts apply to the receptor embodiments.

10   The purified protein or defined peptides are useful for generating antibodies by standard methods, as described above. Synthetic peptides or purified protein can be presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan (1991) <u>Current Protocols in Immunology</u>

15   Wiley/Greene; and Harlow and Lane (1989) <u>Antibodies: A Laboratory Manual</u> Cold Spring Harbor Press. Alternatively, a chemokine or receptor may be used as a specific binding reagent, and advantage can be taken of its specificity of binding, much like an antibody would be used. The chemokine

20   receptors are typically 7 transmembrane proteins, which could be sensitive to appropriate interaction with lipid or membrane. The signal transduction typically is mediated through a G-protein, through interaction with a G-protein coupled receptor.

25   For example, the specific binding composition could be used for screening of an expression library made from a cell line which expresses a particular chemokine. The screening can be standard staining of surface expressed ligand, or by panning. Screening of intracellular expression can also be

30   performed by various staining or immunofluorescence procedures. The binding compositions could be used to affinity purify or sort out cells expressing the ligand.

The peptide segments can also be used to predict appropriate oligonucleotides to screen a library, e.g., to

35   isolate species variants. The genetic code can be used to select appropriate oligonucleotides useful as probes for screening. See, e.g., Figures 1-5. In combination with polymerase chain reaction (PCR) techniques, synthetic

oligonucleotides will be useful in selecting correct clones
from a library. Complementary sequences will also be used as
probes or primers. Anchored vector or poly-A complementary
PCR techniques or complementary DNA of other peptides may be

5   useful. Complementary nucleic acid sequences may also be used
as mutagenesis primers.

This invention contemplates use of isolated DNA or
fragments to encode a biologically active corresponding
chemokine polypeptide. In addition, this invention covers

10   isolated or recombinant DNA which encodes a biologically
active protein or polypeptide which is capable of hybridizing
under appropriate conditions with the DNA sequences described
herein. Said biologically active protein or polypeptide can
be an intact ligand. receptor, or fragment, and have an amino

15   acid sequence as disclosed in Figures 1-5. Further, this
invention covers the use of isolated or recombinant DNA, or
fragments thereof, which encode proteins which are homologous
to a chemokine or receptor or which was isolated using such a
cDNA encoding a chemokine or receptor as a probe. The

20   isolated DNA can have the respective regulatory sequences in
the 5' and 3' flanks, e.g., promoters, enhancers, poly-A
addition signals, and others.

An "isolated" nucleic acid is a nucleic acid, e.g., an
RNA, DNA, or a mixed polymer, which is substantially separated

25   from other components which naturally accompany a native
sequence, e.g., ribosomes, polymerases, and flanking genomic
sequences from the originating species. The term embraces a
nucleic acid sequence which has been removed from its
naturally occurring environment, and includes recombinant or

30   cloned DNA isolates and chemically synthesized analogs or
analogs biologically synthesized by heterologous systems. A
substantially pure molecule includes isolated forms of the
molecule.

An isolated nucleic acid will generally be a homogeneous

35   composition of molecules, but will, in some embodiments,
contain minor heterogeneity. This heterogeneity is typically
found at the polymer ends or portions not critical to a
desired biological function or activity.

A "recombinant" nucleic acid is defined either by its
method of production or its structure. In reference to its
method of production, e.g., a product made by a process, the
process is use of recombinant nucleic acid techniques, e.g.,
5   involving human intervention in the nucleotide sequence,
typically selection or production. Alternatively, it can be a
nucleic acid made by generating a sequence comprising fusion
of two fragments which are not naturally contiguous to each
other, but is meant to exclude products of nature, e.g.,
10   naturally occurring purified forms. Thus, for example,
products made by transforming cells with any unnaturally
occurring vector is encompassed, as are nucleic acids
comprising sequence derived using a synthetic oligonucleotide
process. Such is often done to replace a codon with a
15   redundant codon encoding the same or a conservative amino
acid, while typically introducing or removing a sequence
recognition site. Alternatively, it is performed to join
together nucleic acid segments of desired functions to
generate a single genetic entity comprising a desired
20   combination of functions not found in the commonly available
natural forms. Restriction enzyme recognition sites are often
the target of such artificial manipulations, but other site
specific targets, e.g., promoters, DNA replication sites,
regulation sequences, control sequences, or other useful
25   features may be incorporated by design. A similar concept is
intended for a recombinant, e.g., fusion, polypeptide.
Specifically included are synthetic nucleic acids which, by
genetic code redundancy, encode polypeptides similar to
fragments of these antigens, and fusions of sequences from
30   various different species variants.

A significant "fragment" in a nucleic acid context is a
contiguous segment of at least about 17 nucleotides, generally
at least about 20 nucleotides, more generally at least about
23 nucleotides, ordinarily at least about 26 nucleotides, more
35   ordinarily at least about 29 nucleotides, often at least about
32 nucleotides, more often at least about 35 nucleotides,
typically at least about 38 nucleotides, more typically at
least about 41 nucleotides, usually at least about 44

nucleotides, more usually at least about 47 nucleotides,
preferably at least about 50 nucleotides, more preferably at
least about 53 nucleotides, and in particularly preferred
embodiments will be at least about 56 or more nucleotides,
5   e.g., 60, 65, 75, 85, 100, 120, 150, 200, 250, 300, 400, etc.
Such fragments may have ends which begin and/or end at
virtually all positions, e.g., beginning at nucleotides 1, 2,
3, etc., and ending at, e.g., 300, 299, 298, 287, etc., in
combinatorial pairs. Particularly interesting polynucleotides
10 have ends corresponding to structural domain boundaries.

A DNA which codes for a particular chemokine or receptor
protein or peptide will be very useful to identify genes,
mRNA, and cDNA species which code for related or homologous
ligands or receptors, as well as DNAs which code for
15 homologous proteins from different species. There are likely
homologs in closely related species, e.g., rodents or
primates. Various chemokine proteins should be homologous and
are encompassed herein, as would be receptors. However,
proteins can readily be isolated under appropriate conditions
20 using these sequences if they are sufficiently homologous.
Typically, primate chemokines or receptors are of particular
interest.

This invention further covers recombinant DNA molecules
and fragments having a DNA sequence identical to or highly
25 homologous to the isolated DNAs set forth herein. In
particular, the sequences will often be operably linked to DNA
segments which control transcription, translation, and DNA
replication. Alternatively, recombinant clones derived from
the genomic sequences, e.g., containing introns, will be
30 useful for transgenic studies, including, e.g., transgenic
cells and organisms, and for gene therapy. See, e.g., Goodnow
(1992) "Transgenic Animals" in Roitt (ed.) <u>Encyclopedia of
Immunology</u> Academic Press, San Diego, pp. 1502-1504; Travis
(1992) <u>Science</u> 256:1392-1394; Kuhn, et al. (1991) <u>Science</u>
35 254:707-710; Capecchi (1989) <u>Science</u> 244:1288; Robertson
(1987)(ed.) <u>Teratocarcinomas and Embryonic Stem Cells: A
Practical Approach</u> IRL Press, Oxford; and Rosenberg (1992) <u>J.
Clinical Oncology</u> 10:180-199.

Homologous nucleic acid sequences, when compared, exhibit significant similarity, or identity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon
5   hybridization conditions. The hybridization conditions are described in greater detail below.

Substantial homology in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when
10 optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least about 56%, more generally at least about 59%, ordinarily at least about 62%, more ordinarily at least about 65%, often at least about 68%, more often at least about 71%,
15 typically at least about 74%, more typically at least about 77%, usually at least about 80%, more usually at least about 85%, preferably at least about 90%, more preferably at least about 95 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides. Alternatively,
20 substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, typically using a sequence derived from Figures 1-5. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least
25 about 30 nucleotides, preferably at least about 65% over a stretch of at least about 25 nucleotides, more preferably at least about 75%, and most preferably at least about 90% over about 20 nucleotides. See, Kanehisa (1984) Nuc. Acids Res. 12:203-213. The length of homology comparison, as described,
30 may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least
35 about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides. PCR primers will generally have high levels of matches over potentially shorter lengths.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates

5   are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

10   Optical alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482, by the homology alignment algorithm of Needlman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson

15   and Lipman (1988) Proc. Nat'l Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al.,

20   supra).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a

25   tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (1987) J. Mol. Evol. 35:351-360. The method used is similar to the method described by Higgins and Sharp (1989) CABIOS 5:151-153.

30   The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most

35   related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise

alignments. The program is run by designating specific
sequences and their amino acid or nucleotide coordinates for
regions of sequence comparison and by designating the program
parameters. For example, a reference sequence can be compared
5   to other test sequences to determine the percent sequence
identity relationship using the following parameters: default
gap weight (3.00), default gap length weight (0.10), and
weighted end gaps.

        Another example of algorithm that is suitable for
10 determining percent sequence identity and sequence similarity
is the BLAST algorithm, which is described Altschul, et al.
(1990) J. Mol. Biol. 215:403-410. Software for performing
BLAST analyses is publicly available through the National
Center for Biotechnology Information
15 (http:www.ncbi.nlm.nih.gov/). This algorithm involves first
identifying high scoring sequence pairs (HSPs) by identifying
short words of length W in the query sequence, which either
match or satisfy some positive-valued threshold score T when
aligned with a word of the same length in a database sequence.
20 T is referred to as the neighborhood word score threshold
(Altschul, et al., supra). These initial neighborhood word
hits act as seeds for initiating searches to find longer HSPs
containing them. The word hits are then extended in both
directions along each sequence for as far as the cumulative
25 alignment score can be increased. Extension of the word hits
in each direction are halted when: the cumulative alignment
score falls off by the quantity X from its maximum achieved
value; the cumulative score goes to zero or below, due to the
accumulation of one or more negative-scoring residue
30 alignments; or the end of either sequence is reached. The
BLAST algorithm parameters W, T, and X determine the
sensitivity and speed of the alignment. The BLAST program
uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring
matrix (see Henikoff and Henikoff (1989) Proc. Nat'l Acad.
35 Sci. USA 89:10915) alignments (B) of 50, expectation (E) of
10, M=5, N=4, and a comparison of both strands.

        In addition to calculating percent sequence identity, the
BLAST algorithm also performs a statistical analysis of the

similarity between two sequences (see, e.g., Karlin and
Altschul (1993) <u>Proc. Nat'l Acad. Sci. USA</u> 90:5873-5787). One
measure of similarity provided by the BLAST algorithm is the
smallest sum probability (P(N)), which provides an indication

5   of the probability by which a match between two nucleotide or
amino acid sequences would occur by chance. For example, a
nucleic acid is considered similar to a reference sequence if
the smallest sum probability in a comparison of the test
nucleic acid to the reference nucleic acid is less than about

10   0.1, more preferably less than about 0.01, and most preferably
less than about 0.001.

    A further indication that two nucleic acid sequences of
polypeptides are substantially identical is that the
polypeptide encoded by the first nucleic acid is

15   immunologically cross reactive with the polypeptide encoded by
the second nucleic acid, as described below. Thus, a
polypeptide is typically substantially identical to a second
polypeptide, for example, where the two peptides differ only
by conservative substitutions. Another indication that two

20   nucleic acid sequences are substantially identical is that the
two molecules hybridize to each other under stringent
conditions, as described below.

    Stringent conditions, in referring to homology in the
hybridization context, will be stringent combined conditions

25   of salt, temperature, organic solvents, and other parameters,
typically those controlled in hybridization reactions.
Stringent temperature conditions will usually include
temperatures in excess of about 30° C, more usually in excess
of about 37° C, typically in excess of about 45° C, more

30   typically in excess of about 55° C, preferably in excess of
about 65° C, and more preferably in excess of about 70° C.
Stringent salt conditions will ordinarily be less than about
1000 mM, usually less than about 500 mM, more usually less
than about 400 mM, typically less than about 300 mM,

35   preferably less than about 200 mM, and more preferably less
than about 150 mM, e.g., 20-50 mM. However, the combination
of parameters is much more important than the measure of any
single parameter. See, e.g., Wetmur and Davidson (1968) <u>J.</u>

<u>Mol. Biol.</u> 31:349-370. Hybridization under stringent
conditions should give a background of at least 2-fold over
background, preferably at least 3-5 or more.

5   Corresponding chemokines or receptors from other closely
related species can be cloned and isolated by cross-species
hybridization. Alternatively, sequences from a sequence data
base may be recognized as having similarity. Homology may be
very low between distantly related species, and thus
hybridization of relatively closely related species is
10   advisable. Alternatively, preparation of an antibody
preparation which exhibits less species specificity may be
useful in expression cloning approaches. PCR approaches using
segments of conserved sequences will also be used.

15   VII.   Making Chemokines or Receptors; Mimetics
    DNA which encodes each respective chemokine, receptor, or
fragments thereof can be obtained by chemical synthesis,
screening cDNA libraries, or by screening genomic libraries
prepared from a wide variety of cell lines or tissue samples.
20   A "coding sequence" or a sequence which "encodes" a particular
protein, is a nucleic acid sequence which is transcribed (in
the case of DNA) and translated (in the case of mRNA) into a
polypeptide *in vitro* or *in vivo* when placed under the control
of appropriate regulatory sequences. The boundaries of the
25   coding sequence are determined by a start codon at the 5'
(amino) terminus and a translation stop codon at the 3'
(carboxy) terminus. A transcription termination sequence will
usually be located 3' to the coding sequence.

    This DNA can be expressed in a wide variety of host cells
30   for the synthesis of a full-length ligand or fragments which
can in turn, for example, be used to generate polyclonal or
monoclonal antibodies; for binding studies; for construction
and expression of modified molecules; for expression cloning
or purification; and for structure/function studies. Each
35   protein or its fragments can be expressed in host cells that
are transformed with appropriate expression vectors. These
molecules can be substantially purified to be free of protein

or cellular contaminants, other than those derived from the
recombinant host, and therefore are particularly useful in
pharmaceutical compositions when combined with a
pharmaceutically acceptable carrier and/or diluent. The
5   antigens or antibodies, or portions thereof, may be expressed
as fusions with other proteins.

Expression vectors are typically self-replicating DNA or
RNA constructs containing the desired protein gene or its
fragments, usually operably linked to suitable genetic control
10   elements that are recognized in a suitable host cell. These
control elements are capable of effecting expression within a
suitable host. The specific type of control elements
necessary to effect expression will depend upon the eventual
host cell used. Generally, the genetic control elements can
15   include a prokaryotic promoter system or a eukaryotic promoter
expression control system, and typically include a
transcriptional promoter, an optional operator to control the
onset of transcription, transcription enhancers to elevate the
level of mRNA expression, a sequence that encodes a suitable
20   ribosome binding site, and sequences that terminate
transcription and translation. Thus, the term "control
sequences" or "control elements" refers collectively to
promoter sequences, polyadenylation signals, transcription
termination sequences, upstream regulatory domains, origins of
25   replication, internal ribosome entry sites ("IRES"),
enhancers, and the like, which collectively provide for the
replication, transcription and translation of a coding
sequence in a recipient cell. Not all of these control
sequences need always be present so long as the selected
30   coding sequence is capable of being replicated, transcribed
and translated in an appropriate host cell. Expression
vectors also usually contain an origin of replication that
allows the vector to replicate independently of the host cell.

The vectors of this invention contain DNA which encode
35   embodiments of a chemokine, receptor, or a fragment thereof,
typically encoding a biologically active polypeptide. The DNA
can be under the control of a promoter, such as a mammalian or

viral promoter and can encode a selection marker. This
invention further contemplates use of such expression vectors
which are capable of expressing eukaryotic cDNA coding for
each chemokine or receptor in a prokaryotic or eukaryotic
5   host, where the vector is compatible with the host and where
the eukaryotic cDNA coding for the protein is inserted into
the vector such that growth of the host containing the vector
expresses the cDNA in question. Usually, expression vectors
are designed for stable replication in their host cells or for
10   amplification to greatly increase the total number of copies
of the desirable gene per cell. It is not always necessary to
require that an expression vector replicate in a host cell,
e.g., it is possible to effect transient expression of the
ligand or its fragments in various hosts using vectors that do
15   not contain a replication origin that is recognized by the
host cell. It is also possible to use vectors that cause
integration of a chemokine or receptor gene or its fragments
into the host DNA by recombination, or to integrate a promoter
which controls expression of an endogenous gene. Thus, as
20   used herein, a cell has been "transformed" by exogenous DNA
when such exogenous DNA has been introduced inside the cell
membrane. Exogenous DNA may or may not be integrated
(covalently linked) into chromosomal DNA making up the genome
of the cell. A stably transformed cell is one in which the
25   exogenous DNA has become integrated into the chromosome so
that it is inherited by daughter cells through chromosome
replication. This stability is demonstrated by the ability of
the eucaryotic cell to establish cell lines or clones
comprised of a population of daughter cells containing the
30   exogenous DNA.
     Vectors, as used herein, comprise plasmids, viruses,
bacteriophage, integratable DNA fragments, and other vehicles,
including those which enable the integration of DNA fragments
into the genome of the host. Expression vectors are
35   specialized vectors which contain genetic control elements
that effect expression of operably linked genes. Plasmids are
the most commonly used form of vector but many other forms of

vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, et al. (1985 and Supplements) Cloning Vectors: A Laboratory Manual, Elsevier, N.Y., and Rodriquez, et al.
5   (1988)(eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston, MA.

Transformed cells include cells, preferably mammalian, that have been transformed with a chemokine or receptor gene containing vector constructed using recombinant DNA
10   techniques. Transformed host cells usually express the ligand, receptor, or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the protein. This invention further contemplates culturing transformed cells in a nutrient medium, thus
15   permitting the protein to accumulate in the culture. The protein can be recovered, from the culture or from the culture medium, or from cell membranes.

For purposes of this invention, DNA sequences are operably linked when they are functionally related to each
20   other. For example, DNA for a presequence or secretory signal is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the
25   transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such as repressor genes are not contiguously linked but still bind
30   to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram negative and gram positive organisms, e.g., E. coli and B. subtilis. Lower eukaryotes include yeasts, e.g., S.
35   cerevisiae and Pichia, and species of the genus Dictyostelium. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human,

primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, E. coli and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express these chemokines or their fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See Brosius, et al. (1988) "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in Rodriguez and Denhardt (eds.) <u>Vectors: A Survey of Molecular Cloning Vectors and Their Uses</u>, Buttersworth, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and Dictyostelium, may be transformed with chemokine or receptor sequence containing nucleic acids. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, Saccharomyces cerevisiae. It will be used to generically represent lower eukaryotes although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the desired protein or its fragments, and sequences for translation termination, polyadenylation, and transcription termination. Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are the preferred host cells for expression of the functionally active chemokine

or receptor proteins. In principle, most any higher
eukaryotic tissue culture cell line is workable, e.g., insect
baculovirus expression systems, whether from an invertebrate
or vertebrate source. However, mammalian cells are preferred,
5   in that the processing, both cotranslationally and
posttranslationally, will be typically most like natural.
Transformation or transfection and propagation of such cells
has become a routine procedure. Examples of useful cell lines
include HeLa cells, Chinese hamster ovary (CHO) cell lines,
10 baby rat kidney (BRK) cell lines, insect cell lines, bird cell
lines, and monkey (COS) cell lines. Expression vectors for
such cell lines usually include an origin of replication, a
promoter, a translation initiation site, RNA splice sites (if
genomic DNA is used), a polyadenylation site, and a
15 transcription termination site. These vectors also usually
contain a selection gene or amplification gene. Suitable
expression vectors may be plasmids, viruses, or retroviruses
carrying promoters derived, e.g., from such sources as from
adenovirus, SV40, parvoviruses, vaccinia virus, or
20 cytomegalovirus. Representative examples of suitable
expression vectors include pCDNA1; pCD, see Okayama, et al.
(1985) Mol. Cell Biol. 5:1136-1142; pMC1neo Poly-A, see
Thomas, et al. (1987) Cell 51:503-512; and a baculovirus
vector such as pAC 373 or pAC 610.
25   It will often be desired to express a chemokine or
receptor polypeptide in a system which provides a specific or
defined glycosylation pattern. In this case, the usual
pattern will be that provided naturally by the expression
system. However, the pattern will be modifiable by exposing
30 the polypeptide, e.g., an unglycosylated form, to appropriate
glycosylating proteins introduced into a heterologous
expression system. For example, a chemokine or receptor gene
may be co-transformed with one or more genes encoding
mammalian or other glycosylating enzymes. Using this
35 approach, certain mammalian glycosylation patterns will be
achievable or approximated in prokaryote or other cells.
    A chemokine, receptor, or a fragment thereof, may be
engineered to be phosphatidyl inositol (PI) linked to a cell

membrane, but can be removed from membranes by treatment with a phosphatidyl inositol cleaving enzyme, e.g., phosphatidyl inositol phospholipase-C. This releases the antigen in a biologically active form, and allows purification by standard

5   procedures of protein chemistry. See, e.g., Low (1989) Biochim. Biophys. Acta 988:427-454; Tse, et al. (1985) Science 230:1003-1008; and Brunner, et al. (1991) J. Cell Biol. 114:1275-1283.

       Now that these chemokines and receptors have been

10   characterized, fragments or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young (1984) Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky (1984) The Practice

15   of Peptide Synthesis, Springer-Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-

20   hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexyl-carbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

25   These chemokines, receptors, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence,

30   or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction are typically protected to prevent coupling at an incorrect location.

       If a solid phase synthesis is adopted, the C-terminal

35   amino acid is typically bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it has a binding capability to a reactive carboxyl group. Examples of such

insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxycarbonyl-hydrazidated resins, and the like.

5   An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step by step. After synthesizing the complete sequence, the peptide is split off
10   from the insoluble carrier to produce the peptide. This solid-phase approach is generally described, e.g., by Merrifield, et al. (1963) in J. Am. Chem. Soc. 85:2149-2156.

The prepared ligand and fragments thereof can be isolated and purified from the reaction mixture by means of peptide
15   separation, e.g., by extraction, precipitation, electrophoresis, and various forms of chromatography, and the like. The various chemokines or receptors of this invention can be obtained in varying degrees of purity depending upon its desired use. Purification can be accomplished by use of
20   the protein purification techniques disclosed herein or by the use of the antibodies herein described, e.g., in immunoabsorbant affinity chromatography. This immunoabsorbant affinity chromatography is typically carried out, e.g., by first linking the antibodies to a solid support and then
25   contacting the linked antibodies with solubilized lysates of appropriate source cells, lysates of other cells expressing the ligand or receptor, or lysates or supernatants of cells producing the desired proteins as a result of DNA techniques, see below.

30

VIII. Uses

The present invention provides reagents which will find use in diagnostic applications as described elsewhere herein, e.g., in the general description for developmental
35   abnormalities, or below in the description of kits for diagnosis.

This invention also provides reagents with significant therapeutic potential. These chemokines and receptors

(naturally occurring or recombinant), fragments thereof, and binding compositions, e.g., antibodies thereto, along with compounds identified as having binding affinity to them, should be useful in the treatment of conditions associated

5   with abnormal physiology or development, including inflammatory conditions, e.g., asthma, and epithelial conditions, for example, improper wound healing. In particular, modulation of trafficking of leukocytes is one likely biological activity, but a wider tissue distribution

10   might suggest broader biological activity, including, e.g., antiviral effects. Abnormal proliferation, regeneration, degeneration, and atrophy may be modulated by appropriate therapeutic treatment using the compositions provided herein. For example, a disease or disorder associated with abnormal

15   expression or abnormal signaling by a chemokine or ligand for a receptor should be a likely target for an agonist or antagonist of the ligand.

     Various abnormal physiological or developmental conditions are known in cell types shown to possess the

20   chemokine or receptor mRNAs by Northern blot analysis. See Berkow (ed.) The Merck Manual of Diagnosis and Therapy, Merck & Co., Rahway, N.J.; and Thorn, et al. Harrison's Principles of Internal Medicine, McGraw-Hill, N.Y. Developmental or functional abnormalities, e.g., of the immune system, cause

25   significant medical abnormalities and conditions which may be susceptible to prevention or treatment using compositions provided herein.

     Antibodies to the chemokines or receptors, including recombinant forms, can be purified and then used

30   diagnostically or therapeutically, alone or in combination with other chemokines, cytokines, or antagonists thereof. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g.,

35   immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile filtered and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations.

This invention also contemplates use of antibodies or binding fragments thereof, including forms which are not complement binding. Moreover, modifications to the antibody molecules or antigen binding fragments thereof, may be adopted which affect

5   the pharmacokinetics or pharmacodynamics of the therapeutic entity.

Drug screening using antibodies or receptor or fragments thereof can be performed to identify compounds having binding affinity to each chemokine or receptor, including isolation of

10   associated components. Subsequent biological assays can then be utilized to determine if the compound has intrinsic stimulating activity and is therefore a blocker or antagonist in that it blocks the activity of the ligand. Likewise, a compound having intrinsic stimulating activity can activate

15   the receptor and is thus an agonist in that it simulates the activity of a ligand. This invention further contemplates the therapeutic use of antibodies to these chemokines as antagonists, or to the receptors as antagonists or agonists. This approach should be particularly useful with other

20   chemokine or receptor species variants.

Moreover, the novel chemokines and receptors described herein can be used to effect wound healing, as well as to treat a wide variety of disorders associated with proliferative responses. For example, tissue fibrosis is the

25   formation of excessive amounts of fibrotic or scar tissue. Major organs such as the heart, kidney, liver, eye, and skin are prone to chronic scarring. Hypertrophic scars (non-malignant tissue bulk) are a common form of fibrosis caused by burns and other trauma. In addition, there are a number of

30   other fibroproliferative disorders, including scleroderma, keloids, and atherosclerosis, which are associated with general tissue scarring, tumor-like growths in the skin, or sustained scarring of blood vessels. Fibrosis can follow surgery in the form of adhesions, keloid tumors or

35   hypertrophic scarring. Fibrosis causes contractures and joint dislocation following severe burns, wounds or orthopaedic injuries; it can occur in any organ and accompanies many

disease states, such as hepatitis (liver cirrhosis),
hypertension (heart failure), tuberculosis (pulmonary
fibrosis) and diabetes (nephropathy).

Fibrosis, despite the cause, activates a proliferative
5   response related to the events that occur in the healing of
skin wounds. Specifically, acute physical damage causes the
death of cells, the clotting of blood, an inflammatory
response characterized by the migration of inflammatory cells
into the lesion, the proliferation of fibroblastic cells in
10 the lesion and the production and deposition of collagen
fibers and scars.

As demonstrated herein, BLRx, a representative chemokine
receptor, is expressed by fibroblasts, melanocytes and
keratinocytes and is involved in wound healing. In
15 particular, enhanced expression of BLRx is associated with the
onset of the wound healing process. Thus, the compositions
and methods described herein may be used for treating wounds,
such as burns and lesions, as well as for treating or
preventing any of the various forms of fibrosis described
20 above as the processes involved in fibrosis are the same as
those involved in wound healing. Moreover, the compositions
and methods may be used in the treatment of hyperproliferative
diseases of the epidermis, such as psoriasis and basal cell
carcinoma. The methods and compositions are also useful for
25 stimulation of transplanted corneal tissue.

For example, the compositions and methods described
herein can be used to enhance wound healing, block the
fibrotic process and/or control hyperproliferative disorders
using agents that modulate (i.e., either inhibit or enhance)
30 endogenous BLRx production. In treating these disorders, an
increase in the concentration of endogenous BLRx may be
desired, such as in the case of wound healing. Thus, one
method of treatment includes administering a composition that
enhances BLRx activity, for example, the ability of BLRx to

bind to its ligand, or a composition that enhances endogenous BLRx expression. For example, target cells can be transformed with DNA constructs that either encode BLRx or that include transcriptional and/or translational regulatory elements that

5   enhance expression of endogenous BLRx. The exogenous polynucleotides need not code for exact copies of the endogenous BLRx proteins. Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described for

10   instance, in Sambrook et al.,(1989) <u>Molecular Cloning: A Laboratory Manual</u> (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory. For example, hydroxylamine can also be used to introduce single base mutations into the coding region of the gene (Sikorski et al. (1991) <u>Meth. Enzymol.</u> 194: 302-318).

15   Additionally, an agonist, i.e., a molecule that increases or prolongs the duration of the effect of BLRx, can be administered. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that bind to and modulate the effects of the target molecule.

20   Conversely, a decrease in the concentration or expression of endogenous BLRx may be desired, such as when treating a BLRx-associated proliferative disorder, for example fibrotic or sclerotic disease such as liver sclerosis, lung fibrosis and local/systemic sclerosis, cancer, angiogenesis, and

25   atherosclerosis. In this context, one method of treatment includes the administration of a composition that decreases BLRx activity, or causes a decreased production of endogenous BLRx. Thus, an antagonist, i.e., a molecule which, when bound to BLRx, decreases the extent or duration of the effect of the

30   biological activity, can be administered. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules which decrease the effect of the target molecule. Thus, one method of treatment includes transforming a target cell with a polynucleotide that includes

35   transcriptional and/or translational regulatory elements that decrease or suppress expression of endogenous BLRx. Another

method of treatment includes administering a composition that interferes with the binding of BLRx to its ligand, such as by competitively binding to BLRx or by otherwise decreasing the ability of BLRx to bind to or process its ligand, or by

5   effecting the ability of BLRx to participate in downstream signaling. Thus, in one embodiment of the present invention, methods for treatment of a proliferative disorder involve the administration of a therapeutically effective amount of an antibody which specifically reacts with BLRx. Such antibodies

10   are described in detail above.

In another embodiment, a method of the present invention involves the administration of a therapeutically effective amount of an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of genomic

15   DNA or an mRNA molecule which encodes BLRx, so as to prevent transcripton or translation of BLRx mRNA. By "antisense" is meant a composition containing a nucleic acid sequence which is complementary to the "sense" strand of a specific nucleic acid sequence. Once introduced into a cell, the complementary

20   nucleotides combine with endogenous sequences produced by the cell to form duplexes and to block either transcription or translation. See, e.g., Agrawal, S., ed. (1996) Antisense Therapeutics, Humana Press Inc., Totawa NJ; Alama et al. (1997) Pharmacol. Res. 36:171-178; Crooke, S.T. (1997) Adv.

25   Pharmacol. 40:1-49; and Lavrosky et al. (1997) Biochem. Mol. Med. 62(1):11-22. Antisense sequences can be any nucleic acid material, including DNA, RNA, or any nucleic acid mimics or analogs. See, e.g., Rossi et al. (1991) Antisense Res. Dev. 1:285-288; Pardridge et al. (1995) Proc. Nat. Acad. Sci.

30   92:5592-5596; Nielsen and Haaima (1997) Chem. Soc. Rev. 96:73-78; and Lee et al. (1998) Biochemistry 37:900-1010. Delivery of antisense sequences can be accomplished in a variety of ways, such as through intracellular delivery using a recombinant vector.

35   Antisense oligonucleotides of about 15 to 25 nucleic acid bases are typically preferred as such are easily synthesized

and are capable of producing the desired inhibitory effect.
Molecular analogs of antisense oligonucleotides may also be
used for this purpose and can have added advantages such as
stability, distribution, or limited toxicity advantageous in a
5   pharmaceutical product. In addition, chemically reactive
groups, such as iron-linked ethylenediamine-tetraacetic acid
(EDTA-Fe), can be attached to antisense oligonucleotides,
causing cleavage of the RNA at the site of hybridization.
These and other uses of antisense methods to inhibit the *in*
10   *vitro* translation of genes are well known in the art. See,
e.g., Marcus-Sakura (1988) <u>Anal. Biochem.</u> 172:289.

The delivery of polynucleotides, e.g., for delivering
BLRx genes or antisense oligonucleotides, can be achieved
using recombinant expression vectors, with or without carrier
15   viruses or particles. Such methods are well known in the art.
See, e.g., U.S. Patent Nos. 6,214,804; 6,147,055; 5,703,055;
5,589,466; 5,580,859; Slater et al. (1998) <u>J. Allergy Clin.</u>
<u>Immunol.</u> 102:469-475. For example, delivery of polynucleotide
sequences can be achieved using various viral vectors,
20   including retrovirus and adeno-associated virus vectors. See,
e.g., Miller A.D. (1990) <u>Blood</u> 76:271; and Uckert and Walther
(1994) <u>Pharmacol. Ther.</u> 63:323-347. Vectors which can be
utilized for antisense gene therapy include, but are not
limited to, adenoviruses, herpes viruses, vaccinia, or,
25   preferably, RNA viruses such as retroviruses. Other gene
delivery mechanisms that can be used for delivery of
polynucleotide sequences to target cells include colloidal
dispersion and liposome-derived systems, artificial viral
envelopes, and other systems available to one of skill in the
30   art. See, e.g., Rossi, J.J. (1995) <u>Br. Med. Bull.</u> 51:217-225;
Morris et al. (1997) <u>Nucl. Acids Res.</u> 25:2730-2736; and Boado
et al. (1998) <u>J. Pharm. Sci.</u> 87:1308-1315. For example,
delivery systems can make use of macromolecule complexes,
nanocapsules, microspheres, beads, and lipid-based systems
35   including oil-in-water emulsions, micelles, mixed micelles,
and liposomes.

Site-specific delivery of exogenous genes is also contemplated, such as techniques in which cells are first transfected in culture and stable transfectants are subsequently delivered to the target site. Moreover, delivery

5 can be to the region of the target cell or tissue, i.e., in an area proximal to the tissue to be treated, for example, to the region of the wound.

The quantities of reagents necessary for the various therapies described above will depend upon many different

10 factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy in various populations, including racial subgroups, age, gender, etc. Typically, dosages used

15 *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman, et al.

20 (eds.) (1990) <u>Goodman and Gilman's: The Pharmacological Bases of Therapeutics</u>, 8th Ed., Pergamon Press; and <u>Remington's Pharmaceutical Sciences</u>, 17th ed. (1990), Mack Publishing Co., Easton, Penn.. Methods for administration are discussed therein and below, e.g., for oral, intravenous,

25 intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers typically include water, saline, buffers, and other compounds described, e.g., in the <u>Merck Index</u>, Merck & Co., Rahway, New Jersey. Dosage ranges would ordinarily be expected to be in

30 amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or a slow

35 release apparatus will often be utilized for continuous administration.

A chemokine, fragments thereof, or antibodies to it or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to

5   carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in many conventional dosage formulations. While it is possible for the active ingredient to be administered alone, it is often preferable to present it as a

10   pharmaceutical formulation. Formulations typically comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier should be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients

15   and not injurious to the patient. Carriers may improve storage life, stability, etc. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be

20   presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th ed.

25   (1990), Mack Publishing Co., Easton, Penn.; Avis, et al. (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and Lieberman, et al. (eds.) (1990) Pharmaceutical Dosage Forms:

30   Disperse Systems Dekker, New York. The therapy of this invention may be combined with or used in association with other therapeutic agents. Similar considerations will often apply to receptor based reagents.

       Both the naturally occurring and the recombinant forms of

35   the chemokines or receptors of this invention are particularly useful in kits and assay methods which are capable of screening compounds for binding activity to the proteins. Several methods of automating assays have been developed in

recent years so as to permit screening of tens of thousands of compounds in a short period. See, e.g., Fodor, et al. (1991) Science 251:767-773, which describes means for testing of binding affinity by a plurality of defined polymers

5　synthesized on a solid substrate. The development of suitable assays can be greatly facilitated by the availability of large amounts of purified, soluble chemokine as provided by this invention.

For example, antagonists can normally be found once a

10　ligand has been structurally defined. Testing of potential ligand analogs is now possible upon the development of highly automated assay methods using physiologically responsive cells. In particular, new agonists and antagonists will be discovered by using screening techniques described herein.

15　Viable cells could also be used to screen for the effects of drugs on respective chemokine or G-protein coupled receptor mediated functions, e.g., second messenger levels, i.e., Ca^{++}; inositol phosphate pool changes (see, e.g., Berridge (1993) Nature 361:315-325 or Billah and Anthes (1990) Biochem. J.

20　269:281-291); cellular morphology modification responses; phosphoinositide lipid turnover; an antiviral response. and others. Some detection methods allow for elimination of a separation step, e.g., a proximity sensitive detection system. Calcium sensitive dyes will be useful for detecting Ca^{++}

25　levels, with a fluorimeter or a fluorescence cell sorting apparatus.

Rational drug design may also be based upon structural studies of the molecular shapes of the chemokines, other effectors or analogs, or the receptors. Effectors may be

30　other proteins which mediate other functions in response to ligand binding, or other proteins which normally interact with the receptor. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional

35　NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g.,

Blundell and Johnson (1976) <u>Protein Crystallography</u>, Academic
Press, New York.

     Purified chemokine or receptor can be coated directly
onto plates for use in the aforementioned drug screening
5   techniques, and may be associated with detergents or lipids.
However, non-neutralizing antibodies, e.g., to the chemokines
or receptors can be used as capture antibodies to immobilize
the respective protein on the solid phase.

     Similar concepts also apply to the chemokine receptor
10   embodiments of the invention.

     IX.   Kits

     This invention also contemplates use of chemokine or
receptor proteins, fragments thereof, peptides, binding
15   compositions, and their fusion products in a variety of
diagnostic kits and methods for detecting the presence of
ligand, antibodies, or receptors. Typically the kit will have
a compartment containing a defined chemokine or receptor
peptide or gene segment or a reagent which recognizes one or
20   the other, e.g., binding reagents.

     A kit for determining the binding affinity of a test
compound to a chemokine or receptor would typically comprise a
test compound; a labeled compound, for example an antibody
having known binding affinity for the protein; a source of
25   chemokine or receptor (naturally occurring or recombinant);
and a means for separating bound from free labeled compound,
such as a solid phase for immobilizing the ligand or receptor.
Once compounds are screened, those having suitable binding
affinity to the ligand or receptor can be evaluated in
30   suitable biological assays, as are well known in the art, to
determine whether they act as agonists or antagonists to the
receptor. The availability of recombinant chemokine or
receptor polypeptides also provide well defined standards for
calibrating such assays or as positive control samples.

35   A preferred kit for determining the concentration of, for
example, a chemokine or receptor in a sample would typically
comprise a labeled compound, e.g., antibody, having known
binding affinity for the target, a source of ligand or

receptor (naturally occurring or recombinant) and a means for separating the bound from free labeled compound, for example, a solid phase for immobilizing the chemokine or receptor. Compartments containing reagents, and instructions for use or disposal, will normally be provided.

Antibodies, including antigen binding fragments, specific for the chemokine or receptor, or fragments are useful in diagnostic applications to detect the presence of elevated levels of chemokine, receptor, and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, body fluids, and further can involve the detection of antigens related to the ligand or receptor in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and antigen complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA), and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the primary antibody to a chemokine or receptor or to a particular fragment thereof. Similar assays have also been extensively discussed in the literature. See, e.g., Harlow and Lane (1988) <u>Antibodies: A Laboratory Manual</u>, CSH.

Anti-idiotypic antibodies may have similar uses to diagnose presence of antibodies against a chemokine or receptor, as such may be diagnostic of various abnormal states. For example, overproduction of a chemokine or receptor may result in production of various immunological reactions which may be diagnostic of abnormal physiological states, particularly in various inflammatory or asthma conditions.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or

unlabeled antibody or labeled chemokine or receptor is
provided. This is usually in conjunction with other
additives, such as buffers, stabilizers, materials necessary
for signal production such as substrates for enzymes, and the
5   like. Preferably, the kit will also contain instructions for
proper use and disposal of the contents after use. Typically
the kit has compartments or containers for each useful
reagent. Desirably, the reagents are provided as a dry
lyophilized powder, where the reagents may be reconstituted in
10   an aqueous medium providing appropriate concentrations of
reagents for performing the assay.

The aforementioned constituents of the drug screening and
the diagnostic assays may be used without modification or may
be modified in a variety of ways. For example, labeling may
15   be achieved by covalently or non-covalently joining a moiety
which directly or indirectly provides a detectable signal. In
any of these assays, the ligand, test compound, chemokine,
receptor, or antibodies thereto can be labeled either directly
or indirectly. Possibilities for direct labeling include
20   label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No.
3,645,090) such as peroxidase and alkaline phosphatase, and
fluorescent labels (U.S. Pat. No. 3,940,475) capable of
monitoring the change in fluorescence intensity, wavelength
shift, or fluorescence polarization. Possibilities for
25   indirect labeling include biotinylation of one constituent
followed by binding to avidin coupled to one of the above
label groups.

There are also numerous methods of separating bound from
the free ligand, or alternatively bound from free test
30   compound. The chemokine or receptor can be immobilized on
various matrixes, perhaps with detergents or associated
lipids, followed by washing. Suitable matrixes include
plastic such as an ELISA plate, filters, and beads. Methods
of immobilizing the chemokine or receptor to a matrix include,
35   without limitation, direct adhesion to plastic, use of a
capture antibody, chemical coupling, and biotin-avidin. The
last step in this approach may involve the precipitation of
antigen/antibody complex by any of several methods including

those utilizing, e.g., an organic solvent such as polyethylene
glycol or a salt such as ammonium sulfate. Other suitable
separation techniques include, without limitation, the
fluorescein antibody magnetizable particle method described in
5   Rattle, et al. (1984) Clin. Chem. 30:1457-1461, and the double
antibody magnetic particle separation as described in U.S.
Pat. No. 4,659,678.

Methods for linking proteins or their fragments to the
various labels have been extensively reported in the
10   literature and do not require detailed discussion here. Many
of the techniques involve the use of activated carboxyl groups
either through the use of carbodiimide or active esters to
form peptide bonds, the formation of thioethers by reaction of
a mercapto group with an activated halogen such as
15   chloroacetyl, or an activated olefin such as maleimide, for
linkage, or the like. Fusion proteins will also find use in
these applications.

Another diagnostic aspect of this invention involves use
of oligonucleotide or polynucleotide sequences taken from the
20   sequence of the chemokine or receptor. These sequences can be
used as probes for detecting levels of the ligand message in
samples from patients suspected of having an abnormal
condition, e.g., an inflammatory, physiological, or
developmental problem. The preparation of both RNA and DNA
25   nucleotide sequences, the labeling of the sequences, and the
preferred size of the sequences has received ample description
and discussion in the literature. Normally an oligonucleotide
probe should have at least about 14 nucleotides, usually at
least about 18 nucleotides, and the polynucleotide probes may
30   be up to several kilobases. Various labels may be employed,
most commonly radionuclides, particularly ^{32}P. However, other
techniques may also be employed, such as using biotin modified
nucleotides for introduction into a polynucleotide. The
biotin then serves as the site for binding to avidin or
35   antibodies, which may be labeled with a wide variety of
labels, such as radionuclides, fluorescers, enzymes, or the
like. Alternatively, antibodies may be employed which can
recognize specific duplexes, including DNA duplexes, RNA

duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody

5   bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART).

10 This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of

15 multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, et al. (1989) Progress in Growth Factor Res. 1:89-97.

X.   Receptor for Chemokine; Ligands for Receptors

20   Having isolated a ligand binding partner of a specific interaction, methods exist for isolating the counter-partner. See, Gearing, et al EMBO J. 8:3667-4676 or McMahan, et al. (1991) EMBO J. 10:2821-2832. For example, means to label a chemokine without interfering with the binding to its receptor

25 can be determined. For example, an affinity label can be fused to either the amino- or carboxy-terminus of the ligand. An expression library can be screened for specific binding of chemokine, e.g., by cell sorting, or other screening to detect subpopulations which express such a binding component. See,

30 e.g., Ho, et al. (1993) Proc. Nat'l Acad. Sci. 90:11267-11271. Alternatively, a panning method may be used. See, e.g., Seed and Aruffo (1987) Proc. Nat'l. Acad. Sci. 84:3365-3369.

With a receptor, means to identify the ligand exist. Methods for using the receptor, e.g., on the cell membrane,

35 can be used to screen for ligand by, e.g., assaying for a common G-protein linked signal such as Ca++ flux. See Lerner (1994) Trends in Neurosciences 17:142-146. It is likely that the ligands for these receptors are chemokines.

Protein cross-linking techniques with label can be applied to a isolate binding partners of a chemokine. This would allow identification of protein which specifically interacts with a chemokine, e.g., in a ligand-receptor like

5   manner.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to specific embodiments.

10

EXAMPLES

I.   General Methods

Many of the standard methods below are described or
referenced, e.g., in Maniatis, et al. (1982) <u>Molecular
Cloning, A Laboratory Manual</u> Cold Spring Harbor Laboratory,
Cold Spring Harbor Press, NY; Sambrook, et al. (1989)
<u>Molecular Cloning: A Laboratory Manual</u> (2d ed.) Vols. 1-3, CSH
Press, NY; Ausubel, et al., <u>Biology</u> Greene Publishing
Associates, Brooklyn, NY; or Ausubel, et al. (1987 and
Supplements) <u>Current Protocols in Molecular Biology</u>
Wiley/Greene, NY; Innis, et al. (eds.) (1990) <u>PCR Protocols: A
Guide to Methods and Applications</u> Academic Press, NY. Methods
for protein purification include such methods as ammonium
sulfate precipitation, column chromatography, electrophoresis,
centrifugation, crystallization, and others. See, e.g.,
Ausubel, et al. (1987 and periodic supplements); Deutscher
(1990) "Guide to Protein Purification," <u>Methods in Enzymology</u>
vol. 182, and other volumes in this series; Coligan, et al.
(1995 and supplements) <u>Current Protocols in Protein Science</u>
John Wiley and Sons, New York, NY; P. Matsudaira (ed.) (1993)
<u>A Practical Guide to Protein and Peptide Purification for
Microsequencing</u>, Academic Press, San Diego, CA; and
manufacturer's literature on use of protein purification
products, e.g., Pharmacia, Piscataway, NJ, or Bio-Rad,
Richmond, CA. Combination with recombinant techniques allow
fusion to appropriate segments (epitope tags), e.g., to a FLAG
sequence or an equivalent which can be fused, e.g., via a
protease-removable sequence. See, e.g., Hochuli (1989)
<u>Chemische Industrie</u> 12:69-70; Hochuli (1990) "Purification of
Recombinant Proteins with Metal Chelate Absorbent" in Setlow
(ed.) <u>Genetic Engineering, Principle and Methods</u> 12:87-98,
Plenum Press, NY; and Crowe, et al. (1992) <u>QIAexpress: The
High Level Expression & Protein Purification System</u> QUIAGEN,
Inc., Chatsworth, CA.

Standard immunological techniques are described, e.g., in
Hertzenberg, et al. (eds. 1996) <u>Weir's Hanbook of Experimental
Immunology</u> vols 1-4, Blackwell Science; Coligan (1991) <u>Current</u>

Protocols in Immunology Wiley/Greene, NY; and Methods in
Enzymology volumes. 70, 73, 74, 84, 92, 93, 108, 116, 121,
132, 150, 162, and 163. Assays for neural cell biological
activities are described, e.g., in Wouterlood (ed. 1995)

5   Neuroscience Protocols modules 10, Elsevier; Methods in
Neurosciences Academic Press; and Neuromethods Humana Press,
Totowa, NJ. Methodology of developmental systems is
described, e.g., in Meisami (ed.) Handbook of Human Growth and
Developmental Biology CRC Press; and Chrispeels (ed.)

10   Molecular Techniques and Approaches in Developmental Biology
Interscience.

         FACS analyses are described in Melamed, et al. (1990)
Flow Cytometry and Sorting Wiley-Liss, Inc., New York, NY;
Shapiro (1988) Practical Flow Cytometry Liss, New York, NY;

15   and Robinson, et al. (1993) Handbook of Flow Cytometry Methods
Wiley-Liss, New York, NY.

II.   Isolation and characterization of rodent CXC N4 cDNAs
         The rodent CXC N4 was identified from a mouse cDNA

20   library. Individual cDNA clones are sequenced using
standard methods, e.g., the Taq DyeDeoxy Terminator Cycle
Sequencing kit (Applied Biosystems, Foster City, CA), and
the sequence is further characterized.

         The predicted signal sequence corresponds to amino

25   acids met1 to about gly19, so the mature form should begin
with gln20 and run about the standard chemokine length,
e.g., about 90 to 110 residues. Additional processing may
occur in a physiological system.

         Computer analysis and alignments for related genes

30   indicates the closest match is to the mouse chemokines
SDF-1, IP-10, and MIG. This similarity in sequence may
well correlate with similarity in regulation, which
suggests related functions. The absence of the ELR motif
in the CXC chemokine suggests that it will not bind to the

35   IL-8 receptors, and is probably not angiogenic. It may
still be angiostatic, suggesting some possible use in
tumor or related therapies. Conversely, an antagonist is
likely to block its activity, which suggests therapeutic

or research use where angiostasis is undesirable, e.g., wound healing, etc.

Other rodent counterparts should be isolatable using the entire coding portion of this clone as a hybridization probe. A Southern blot or PCR analysis may indicate the extent of homology across species, and either a cDNA library or mRNA can be screened to identify an appropriate cell source. The physiological state of many different cell types may also be evaluated for increased expression of the gene.

III.   Isolation and characterization of GPCR cDNAs

A.   Rodent DNAXCCR10

The partial rodent DNAXCCR10 clone was derived from mouse cDNA library. The nucleotide sequence is provided in Figure 5 (SEQ ID NOS:5 and 6), encoding a polypeptide of about 75 amino acids, at the carboxy terminus of the natural gene.

Computer analysis suggests that the closest related genes are various G-protein coupled receptors. These include the chemokine receptors, and protease, e.g., thrombin, receptors. Structural motifs suggest that the receptor contains motifs characteristic of the chemokine receptor family, and of the protease receptor family. The transmembrane segments, based upon hydrophobicity plots and comparisons with other similar GPCRs, particularly the human and canine GPCR W genes, should be about as follows: TM6 ends at gln2; and TM7 from phe14 to gln36. See, e.g., Loetscher, et al. (1996) J. Expt'l Med. 184:963-969. The amino terminal segment is probably an extracellular segment (E1), and the others would be E2 between TM2 and TM3; E3 between TM4 and TM5; and E4 between TM6 and TM7. The intracellular segments should then run I1 between TM1 and TM2; I2 between TM3 and TM4, I3 between TM5 and TM6, and I4 the carboxy terminus from the end of TM7.

Additional processing may occur in a physiological system. A computer analysis of GPCR sequences will indicate residues characteristic of the family members.

Other rodent counterparts should be isolatable using the entire coding portion of this mouse clone as a hybridization probe. A Southern blot may indicate the extent of homology across species, and either a cDNA

5   library or mRNA can be screened to identify an appropriate cell source. The physiological state of many different cell types may also be evaluated for increased expression of the gene.

Screening for response to various chemokine ligands

10   indicates that the receptor responds to the presence of the MIP-3α chemokine. See Hieshima, et al. (1997) <u>J. Biol. Chem.</u> 272:5846-5853; Hromas, et al. (1997) <u>Blood</u> 89:3315-3322; and Baba, et al. (1997) <u>J. Biol. Chem.</u> 272:14893-14898. The receptor induced a Ca++ flux upon

15   transfection into various cell types and contacting with the MIP-3α. This receptor is distributed on T cells, as discussed below.

This suggests that the MIP-3α chemokine ligand, or its antagonist, should have activity in recruiting resting

20   T cells and/or NK cells to sites of inflammation. It may also be useful in vaccines, exhibiting certain adjuvant effects. Thus, these reagents may be useful in regulation of inflammation, or initiation of an immune response.

B.   Primate BLRx

25   The primate BLRx clone was derived from a cDNA library made from human sequence, including both olfactory epithelium and tonsil cells.

Computer analysis suggests that the closest related genes are various G-protein coupled receptors. Structural

30   analysis indicates that the receptor contains motifs characteristic of the chemokine receptor family, and exhibits similarity to a bovine gustative receptor. The transmembrane segments, based upon hydrophobicity plots and comparisons with other similar GPCRs, should be about

35   as follows: TM1 from val42 to tyr69; TM2 from val78 to val102; TM3 from ile114 to ile135; TM4 from trp155 to val174; TM5 from ala198 to tyr220; TM6 from lys240 to

asn257; TME7 from gln286 to phe305.

Other primate counterparts should be isolatable using the entire coding portion of this human clone as a hybridization probe. A Southern blot may indicate the extent of homology across species, and either a cDNA library or mRNA can be screened to identify an appropriate cell source. The physiological state of many different cell types may also be evaluated for increased expression of the gene.

IV.   Preparation of antibodies

Many standard methods are available for preparation of antibodies. For example, synthetic peptides may be prepared to be used as antigen, administered to an appropriate animal, and either polyclonal or monoclonal antibodies prepared. Short peptides, e.g., less than about 10 amino acids may be expressed as repeated sequences, while longer peptides may be used alone or conjugated to a carrier. For example, with the GPCRs, animals are immunized with peptides or complete proteins from apprpriate portions of Figures 1-5. Highest specificity will result when the polypeptides are selected from portions which are most unique, e.g., not from conserved sequence regions. The animals may be used to collect antiserum, or may be used to generate monoclonal antibodies.

Antiserum is evaluated for use, e.g., in an ELISA, and will be evaluated for utility in immunoprecipitation, e.g., typically native, or Western blot, e.g., denatured antigen, analysis. Monoclonal antibodies will also be evaluated for those same uses.

The antibodies provided will be useful as immunoaffinity reagents, as detection reagents, for immunohistochemistry, and as potential therapeutic reagents, either as agonist or antagonist reagents.

V.   Assays for chemotactic activity of chemokines

Chemokine proteins are produced, e.g., in COS cells

transfected with a plasmid carrying the chemokine cDNA by electroporation. See, Hara, et al. (1992) <u>EMBO J.</u> 10:1875-1884. Physical analytical methods may be applied, e.g., CD analysis, to compare tertiary structure to other

5   chemokines to evaluate whether the protein has likely folded into an active conformation. After transfection, a culture supernatant is collected and subjected to bioassays. A mock control, e.g., a plasmid carrying the luciferase cDNA, is used. See, de Wet, et al. (1987) <u>Mol.</u>

10 <u>Cell. Biol.</u> 7:725-757. A positive control, e.g., recombinant murine MIP-1α from R&D Systems (Minneapolis, MN), is typically used. Likewise, antibodies may be used to block the biological activities, e.g., as a control.

Lymphocyte migration assays are performed as

15 previously described, e.g., in Bacon, et al. (1988) <u>Br. J.</u> <u>Pharmacol.</u> 95:966-974. Murine Th2 T cell clones, CDC-25 (see Tony, et al. (1985) <u>J. Exp. Med.</u> 161:223-241) and HDK-1 (see Cherwinski, et al. (1987) <u>J. Exp. Med.</u> 166:1229-1244), made available from R. Coffman and A.

20 O'Garra (DNAX, Palo Alto, CA), respectively, are used as controls.

Ca2+ flux upon chemokine stimulation is measured, e.g., according to the published procedure described in Bacon, et al. (1995) <u>J. Immunol.</u> 154:3654-3666.

25 Maximal numbers of migrating cells in response to the chemokine being tested are measured. See Schall (1993) <u>J.</u> <u>Exp. Med.</u> 177:1821-1826. A dose-response curve is determined, preferably giving a characteristic bell shaped dose-response curve.

30 After stimulation with various chemokines, lymphocytes often exhibit a measurable intracellular Ca2+ flux. MIP-1α, e.g., is capable of inducing immediate transients of calcium mobilization. Typically, the levels of chemokine used in these assays will be comparable to

35 those used for the chemotaxis assays (1/1000 dilution of conditioned supernatants).

Retroviral infection assays have also been described, and recent description of certain chemokine receptors in retroviral infection processes may indicate that similar roles may apply these receptors. See, e.g., Balter (1996)
5   Science 272:1740 (describing evidence for chemokine receptors as coreceptors for HIV); and Deng, et al. (1996) Nature 381:661-666.

For receptors, biological activity may be measured in response to an appropriate ligand. The receptors are
10 transfected into an assortment of cell types, each of which is likely to possess the intracellular signaling components compatible with the expressed receptor. Various ligand sources are tested to find a source of ligand which results in a G-protein coupled response.
15 Alternatively, the cells are tested for Ca++ flux in response to such ligands. Flux may be conveniently measured by electrophysiological means, or by Ca++ sensitive dyes.

20 VI.   Analysis of individual variation

From the distribution data, an abundant easily accessible cell type is selected for sampling from individuals. Using PCR techniques, a large population of individuals are analysed for this gene. cDNA or other PCR
25 methods are used to sequence the corresponding gene in the different individuals, and their sequences are compared. This indicates both the extent of divergence among racial or other populations, as well as determining which residues are likely to be modifiable without dramatic
30 effects on function.

VII. Biological activities, direct and indirect

A robust and sensitive assay is selected as described above, e.g., on immune cells, neuronal cells, or stem
35 cells. Chemokine is added to the assay in increasing doses to see if a dose response is detected. For example, in a proliferation assay, cells are plated out in plates. Appropriate culture medium is provided, and chemokine is

added to the cells in varying amounts. Growth is
monitored over a period of time which will detect either a
direct effect on the cells, or an indirect effect of the
chemokine.

5   Alternatively, an activation assay or attraction
assay is used. An appropriate cell type is selected, e.g,
hematopoietic cells, myeloid (macrophages, neutrophils,
polymorphonuclear cells, etc.) or lymphoid (T cell, B
cell, or NK cells), neural cells (neurons, neuroglia,
10   oligodendrocytes, astrocytes, etc.), or stem cells, e.g.,
progenitor cells which differentiate to other cell types,
e.g., gut crypt cells and undifferentiated cell types.
        Other assays will be those which have been
demonstrated with other chemokines. See, e.g., Schall and
15   Bacon (1994) Current Opinion in Immunology 6:865-873; and
Bacon and Schall (1996) Int. Arch. Allergy & Immunol.
109:97-109.

VIII.   Structure activity relationship
20   Information on the criticality of particular residues
is determined using standard procedures and analysis.
Standard mutagenesis analysis is performed, e.g., by
generating many different variants at determined
positions, e.g., at the structural positions identified
25   above, and evaluating biological activities of the
variants. This may be performed to the extent of
determining positions which modify activity, or to focus
on specific positions to determine the residues which can
be substituted to either retain, block, or modulate
30   biological activity.
        Alternatively, analysis of natural variants can
indicate what positions tolerate natural mutations. This
may result from populational analysis of variation among
individuals, or across strains or species. Samples from
35   selected individuals are analyzed, e.g., by PCR analysis
and sequencing. This allows evaluation of population
polymorphisms.

IX.   Chromosomal localization

The cDNA is labeled, e.g., nick-translated with biotin-14 dATP and hybridized *in situ* at a final concentration of 5 ng/μl to metaphases from two normal

5   males.   Fluorescence *in situ* hybridization (FISH) method may be modified from that described by Callen, et al. (1990). Ann. Genet. 33:219-221, in that chromosomes are stained before analysis with both prodidium iodide (as counter stain) and DAPI (for chromosome identification).

10   Images of metaphase preparations are captured by a CCD camera and computer enhanced.   Identification of the appropriate labeled chromosomes is determined. Localization to the standard locations for such molecule, or different location may also provide information as to

15   function.

X.   Expression analysis of chemokine/receptor genes

RNA blot and hybridization are performed according to the standard methods in Maniatis, et al. (1982) Molecular

20   Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.   An appropriate fragment or the whole coding sequence of a cDNA fragment is selected for use as a probe.   To verify the amount of RNA loaded in each lane, the substrate membrane is reprobed with a

25   control cDNA, e.g., glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (Clontech, Palo Alto CA).

Analysis of mRNA from the appropriate cell source using the probe will determine the natural size of message.   It will also indicate whether different sized

30   messages exist.   The messages will be subject to analysis after isolation, e.g., by PCR or hybridization techniques.

Northern blot analysis may be performed on many different mRNA sources, e.g., different tissues, different species, or cells exhibiting defined physiological

35   responses, e.g., activation conditions or developmental conditions.   However, in certain cases, cDNA libraries may be used to evaluate sources which are difficult to prepare.   A "reverse Northern" uses cDNA inserts removed

from vector, but multiplicity of bands may reflect either different sized messages, or may be artifact due to incomplete reverse transcription in the preparation of the cDNA library. In such instances, verification may be

5   appropriate by standard Northern analysis.

Similarly, Southern blots may be used to evaluate species distribution of a gene. The stringency of washes of the blot will also provide information as to the extent of homology of various species counterparts.

10   Tissue distribution, and cell distribution, may be evaluated by immunohistochemistry using antibodies. Alternatively, *in situ* nucleic acid hybridization may also be used in such analysis. Certain distribution data may be ascertained by the frequency and tissue types where

15   messages have been found and collected in sequence databases, e.g., GenBank or proprietary collections.

A.   Rodent CXC N4

The mouse CXC N4 sequence was identified from a mouse sequence data base. There is little distribution data

20   generated at this time.

B.   Rodent DNAXCCR10

The rodent DNAXCCR10 was isolated from a mouse cDNA library. A human counterpart nucleic acid is expressed in activate monocytes, and in spleen, lymph node, and

25   appendix, with lesser signals detected in thymus and testis. The signal in testis may be artifact, and should probably be retested under more stringent conditions of isolated tissue. The message appears to be down-regulated in activated PBMC, in activated splenocytes, and activated

30   T cell clones. The message appears to be up-regulated in activated NK cells. It appears up-regulated in activated monocytes, but down-regulated in IL-10 treated monocytes.

The distribution of the receptor points more to the function of the ligand, MIP-3α, as discussed above.

35   C.   Primate BLRx

The BLRx GPCR was identified from primate cDNA library from Weizman Olfactory Epithelium and human

tonsilar cells enriched for germinal center B cells. A
full length clone was isolated from human spleen, and
fragments have been identified in cDNA libraries derived
from endometrium stromal cells and synovial fibroblasts.

5   The message has been detected in cDNA libraries from
liver, brain, gall bladder, small intestine, ovary,
uterus, spleen, and tonsil. A weak signal was detected in
activated monocytes, but this needs to be confirmed.
Message has also been detected in various T cell lines and

10   a CHA cell line, but not in the tested Th1, Th2, or Th0 T
cell clones. A signal was not detected in PBMC. There
appear to be short sequences available from mouse
sequences which correspond to a rodent counterpart for
this gene.

15

XI.   Biological Activity of BLRx
      A.   Expression Analysis
         In order to identify cells types that express BLRx, a
large panel of cDNA libraries was analyzed for the

20   expression of BLRx using quantitative PCR. In particular,
cDNA libraries from various cellular sources (indicated in
Figure 6) were prepared as described previously (Bolin, et
al. (1997) J. Neurosci. 17:5493) and used as templates for
Taqman-PCR analyses. Cells were stimulated with various

25   cytokines as indicated in Figure 6. The cDNAs (50 ng per
reaction) were analyzed for the expression of BLRx genes
by the Fluorogenic 5'-nuclease PCR assay (Holland, et al.
(1991) Proc. Natl. Acad. Sci. USA 88:7276), using an ABI
Prism 7700 Sequence Detection System (Perkin Elmer, Foster

30   City, CA). Reactions were incubated for 2 min at 50 °C,
denatured for 10 min at 95 °C and subjected to 40 two-step
amplification cycles with annealing/extension at 60 °C for
1 min followed by denaturation at 95 °C for 15 sec. The
PCR products were analyzed with FAM-labeled probes.

35   Values were expressed as fg/50 ng total cDNA.
         Results of the expression analysis are presented in
Figure 6. In particular, expression analysis indicated
that BLRx was constitutively expressed in dermal

fibroblasts (FB), with expression significantly down-regulated by TNF-α and IL-1β. IFN-γ slightly down-regulated expression by fibroblasts, while IL-4 and IL-10 had no significant effects on expression levels.

5   BLRx was expressed at lower levels in keratinocytes (KC), melanocytes (MC) and dermal microvascular endothelial cells (DMEC).

These results indicate that BLRx may play a role in wound healing, sclerotic processes, keloid formation,

10   collagen synthesis, scleroderma, systemic sclerosis or in other biological process where these cell types are implicated.

B.   *In Vivo* Wound Healing Studies

15   In order to test the hypothesis that BLRx is involved in the wound healing process, the following experiment was conducted. Groups of female BALB/c mice (3 animals per group) were anesthetized with an intraperitoneal injection of Ketamine and Zylazine. The dorsal region was shaved

20   and the surgical area disinfected. A 2 cm dorsal incisional wound was made through the epidermis and dermis leaving the subcutaneous muscle layer undisturbed. The wound was closed and the wound and surrounding area covered. Animals were sacrificed at 12 hours, 1, 2, 3, 5,

25   7 and 10 days following incision.

Wounds were harvested and RNA was extracted using the RNA STAT 60 method (Tel-Test, New Jersey) and BLRx expression analyzed using Taqman-PCR analysis, as described above. Results are presented in Figure 7.

30   Values are expressed as fg/25 ng total cDNA. As can be seen, BLRx expression is up-regulated during early wound healing, peaking at 12 hours. The results evidence that BLRx plays a role in wound healing.

35   XII. Screening for receptor/ligand

Labeled reagent is useful for screening of an expression library made from a cell line which expresses a chemokine or receptor, as appropriate. Standard staining

techniques are used to detect or sort intracellular or surface expressed ligand, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by various staining
5   or immunofluorescence procedures. See also, e.g., McMahan, et al. (1991) <u>EMBO J.</u> 10:2821-2832.

     For example, on day 0, precoat 2-chamber permanox slides with 1 ml per chamber of fibronectin, 10 ng/ml in PBS, for 30 min at room temperature. Rinse once with PBS.
10 Then plate COS cells at 2-3 x 10^5 cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

     On day 1 for each sample, prepare 0.5 ml of a solution of 66 μg/ml DEAE-dextran, 66 μM chloroquine, and 4 μg DNA in serum free DME. For each set, a positive
15 control is prepared, e.g., of mMIG-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml
20 growth medium and incubate overnight.

     On day 2, change the medium. On days 3 or 4, the cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde (PFA)/glucose for 5 min. Wash 3X with
25 HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations are performed as follows. Add HBSS/saponin(0.1%) with 32 μl/ml of 1M NaN_3 for 20 min. Cells are then washed with HBSS/saponin 1X. Add antibody complex to cells and
30 incubate for 30 min. Wash cells twice with HBSS/saponin. Add second antibody, e.g., Vector anti-mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., Vector Elite ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop
35 of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash cells

twice with HBSS, second wash for 2 min, which closes
cells. Then add Vector diaminobenzoic acid (DAB) for 5 to
10 min. Use 2 drops of buffer plus 4 drops DAB plus 2
drops of H_2O_2 per 5 ml of glass distilled water.

5   Carefully remove chamber and rinse slide in water. Air
dry for a few minutes, then add 1 drop of Crystal Mount
and a cover slip. Bake for 5 min at 85-90° C.

       Alternatively, the binding compositions are used to
affinity purify or sort out cells expressing the ligand or
10   receptor. See, e.g., Sambrook, et al. or Ausubel et al.

       Many modification an variations of this invention can
be made without departing from its spirit and scope, as
will be apparent to those skilled in the art. The
specific embodiments described herein are offered by way
15   of example only, and the invention is to be limited only
by the terms of the appended claims, along with the full
scope of the equivalents to which such claims are
entitled.